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Immunocytochemical Localization of Gonadotropins, Growth Hormone, and Prolactin in Equine Pituitary.

Mohammad Saeed Rahmanian
Louisiana State University and Agricultural & Mechanical College

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IMMUNOCYTOCHEMICAL LOCALIZATION OF GONADOTROPINS, GROWTH HORMONE, AND PROLACTIN IN EQUINE PITUITARY

A Dissertation

Submitted to the Graduate Faculty of the
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in

The Department of Animal Science

by
Mohammad Saeed Rahmanian
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M.S., University of Wyoming 1986
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QUOTATION

"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going direct to Heaven, we were all going direct the other way..."

A Tale of Two Cities
Chapter 1
The Period
Charles Dickens

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The present research was performed to characterize immunoreactive (ir) pituitary cells in the equine species. Two experiments were performed to measure the prevalence of single- and double-hormone labeled gonadotropes, lactotropes, somatotropes, and mammosomatotropes. In Experiments 1 and 2, pituitaries from three intact pony mares were processed for ultrastructural evaluation of cells stained for luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), and growth hormone (GH). Primary antibodies and gold-conjugated antibodies were used to double-stain ir-cells. The percentages of pituitary cells stained for each hormone in intact mares were: total gonadotropes, 15 to 32 %; LH-only, 2 to 16%; FSH-only, 1 to 5%; LH/FSH, 6 to 24%; GH-only, 11 to 26%; PRL-only, 5 to 16%; and GH/PRL, 7 to 17%. In Experiments 3 and 4, long-term ovariectomized (OVX) mares (n = 4/group) were injected with vehicle, estradiol (35 μg/kg), or dihydrotestosterone (DHT; 150 μg/kg) for 21 days. Immunoreactive-cells were identified immunocytochemically and pituitary content of LH, FSH, GH, and PRL and plasma concentrations of LH, FSH, and PRL were measured. DHT reduced ($P < .05$) plasma LH and FSH but had no effect ($P > .1$) on plasma PRL. Estradiol reduced ($P < .05$) plasma FSH but had no effect ($P > .1$) on plasma LH. The percentages of pituitary cells which stained for each hormone in control OVX mares were: total gonadotropes, 12%; LH/FSH, 9%; LH- or FSH-only, 2%; total lactotropes, somatotropes, and mammosomatotropes, 50%; GH-only, 22%; PRL-only, 23%; and GH/PRL, 6%. Estradiol treatment increased ($P < .05$) pituitary concentrations of LH, FSH, and PRL. Estradiol also reduced ($P < .05$) the number of cells stained for

LH/FSH and increased ($P < .05$) the number of lactotropes and mammosomatotropes. DHT reduced ($P < .05$) pituitary concentrations of LH and FSH but did not significantly affect tissue concentrations of PRL or GH. DHT reduced ($P < .05$) the number of cells that stained for LH/FSH and it increased ($P<.05$) the number of FSH-only cells. It is concluded that equine pituitary gonadotropes are subdivided into cells which contain one or both gonadotropins; and PRL and GH also, can be found in separate cells or in same secretory granules within one cell. Furthermore, OVX and steroid treatment preferentially regulate hormone secretion from gonadotropes that produce both LH and FSH as well as the number of ir-lactotropes and mammosomatotropes.

CHAPTER I

INTRODUCTION

Literature in the area of equine endocrinology and reproductive physiology is scarce and lags behind that of other domesticated species. However, in reality, horses could be ideal models to study various aspects of reproductive physiology and endocrinology. One of the unique features of the equine reproductive cycle is the prolonged estrus period (about 7 days) which would provide a window to study the ovarian and pituitary interactions during this phase of the estrous cycle. By treating animals with exogenous steroids and/or hypothalamic factors, one could also shed light on the nature of pituitary cellular responses to various humoral factors.

Another rather unique feature of the equine reproductive cycle is reflected in humoral changes that occur during different seasons (i.e. ovulatory versus anovulatory season) and the possible regulatory role of hormones, such as prolactin (PRL) in seasonal reproductive patterns. Growth hormone (GH), which may not be considered as a reproductive hormone, has recently been getting some attention and its possible interaction with prolactin during different physiological states is being studied. Basic morphological information on the gland that produces these hormones (i.e., the pituitary) and data on the effect of ovarian steroids on morphology and immunoreactive (ir)-staining of pituitary cells do not exist for the equine species. Therefore, the research projects in this dissertation were performed to provide the basic information on subsets of pituitary cells in intact and ovariectomized (OVX) pony mares and to study the effect of steroid treatments on morphology and ir-staining of hormones in pituitary cell populations.

CHAPTER II

LITERATURE REVIEW

The Hypophysis (Pituitary Gland)

Figure 2.1 illustrates the subdivisions of the equine pituitary, which can be divided into a glandular (adenohypophysis) and a neural portion (neurohypophysis). The glandular portion is subdivided into the pars distalis, pars intermedia, and pars tuberalis. The neural portion can be subdivided into the infundibulum/median eminence and pars nervosa [2.1, 2.2].

Adenohypophysis (Anterior Pituitary)

The adenohypophysis (AP) is composed of a solid mass of tightly packed epithelial cells which have been divided into three groups based on their histological staining characteristics: chromophobes, acidophils, and basophils. Prolactin and GH are secreted by the acidophils; luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) are secreted by basophils; and chromophobes do not produce or contain ir-hormones. Corticotropes, which secrete adrenocorticotropic hormone and melanocyte stimulating hormone, are also considered to be basophilic. The carbohydrate moieties on hormones produced by basophils are responsible for the histological staining characteristics of this cell type [2.1, 2.2].

Pars Distalis. Once developed, the pars distalis (PD) is composed of secretory and non-secretory epithelial cells, a connective tissue stroma, and many fenestrated capillaries. The PD accounts for most of the mass of the AP. Despite its close proximity to the brain, the PD contains only a few nerve terminals related to the limited number of

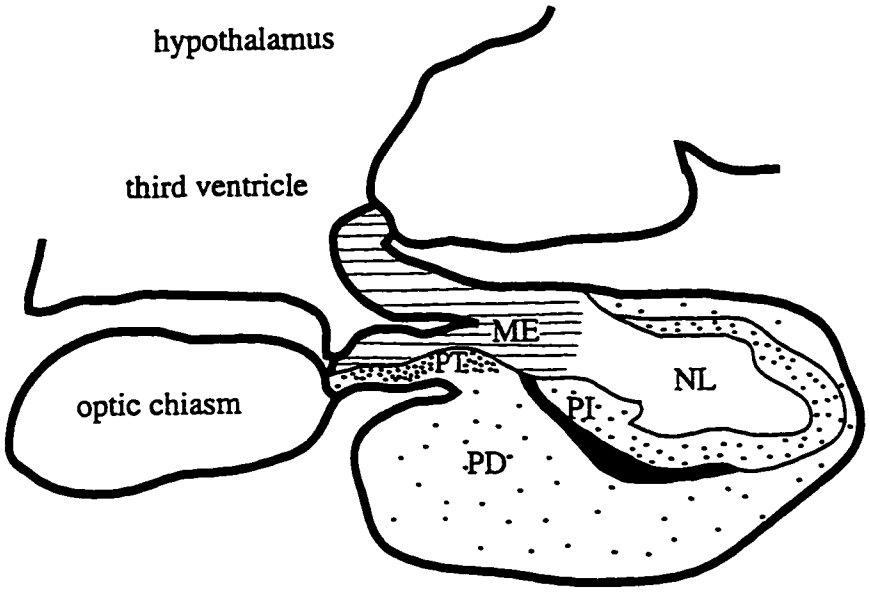

Figure 2.1. Schematic illustration of the equine pituitary. PD = Pars distalis, PI = Pars intermedia, PT = Pars tuberalis, NL = Neural lobe, and ME = Median eminence. Adapted from Melrose PA, unpublished, with permission.

axons that enter the AP along the blood vessels [2.1]. The non-secretory cells in PD are called folliculostellate cells. These stellate-shaped cells have long cytoplasmic processes that extend between neighboring granulated cells. Studies have shown that gap junctions are present only between folliculostellate cells and granulated cells [2.1, 2.2]. Soji and Herbert [2.3], on the basis of freeze fracture electron microscopic studies, proposed that the folliculostellate cells form a network that serves as a syncytium to permit rapid communication between cells that lack innervation.

There is no blood-brain barrier in the PD [2.2, 2.4]. The presence of fenestrated capillaries and the organization of secretory epithelial cells in the PD allows hypothalamic hormones to freely diffuse into the nearby capillaries for subsequent delivery to cords and clusters of pituitary cells immediately adjacent to AP capillaries. Thus, hormones released from neurons in the basal forebrain are rapidly delivered to AP cells in order to allow for almost simultaneous release of various hypothalamic and pituitary hormones. Anatomy of the PD also facilitates exposure of pituitary cells to hormones from peripheral endocrine glands. Furthermore, organization of the PD allows hormone (s) produced by various PD cells to have paracrine effects on pituitary hormone secretion [2.4]. Due to the presence of gap junctions between adjacent AP cells, ions and hormones may diffuse between different hormone secreting cell types [2.3, 2.5, 2.6, 2.7]. Therefore, the anatomy of PD facilitates hormonal, paracrine, and ionic communication.

Pars Intermedia. The pars intermedia (PI) is prominent in horses. The PI in the human pituitary is a poorly developed, rudimentary structure with no apparent endocrine significance, except in pregnant women and the human fetus. The PI is less vascularized

than the PD [2.8]. In the rat's PI, capillaries join with the capillaries of the PD [2.8]. These vascular arrangements form the short portal plexus which functions to deliver products of the PI to the neural lobe and PD or to deliver secretions from the neural lobe and PD to the PI [2.9].

Axons terminate in the PI and make synaptoid contact with epithelial cells, and hence PI cells can be regulated by direct neural contact. Therefore, epithelial cells of the PI can be regulated by long portal routes from the median eminence via the PD, "short" portal routes from the neural lobe, and synaptic contact [2.2].

Pars Tuberalis. This segment of the AP is comprised of glandular epithelial cells arranged in cords and containing dense cytoplasmic granules. Pars tuberalis (PT)-specific cells and follicular cells are found in this subdivision of the AP [2.10]. Pars tuberalis-specific cells are spheroid in shape and are characterized by the presence of only a few secretory granules located at the vascular pole of the cell. These granules are generally smaller than those found in PD cells. Varying numbers of glycogen granules are present in the cytoplasm and these are a distinguishing characteristic of PT-specific cells. Follicular cells, in the human fetal pituitary, have irregular heterochromatic nuclei, no secretory granules, and long processes that extended between secretory cells. At the surface, the follicular cells are lined with microvilli and cilia. Occasionally, an axon terminal is found adjacent to a glandular epithelial cell [2.10]. Gonadotropes have been localized within the PT of the rat [2.11] and in the PT of the monkey (*Macacca mulatta*) [2.12, 2.13]. Girod et al. [2.13] reported localization of β-LH in basophilic cells of the PT in *Macacca irus*. Following hypophysectomy of rats, there was an increase in the

number and size of ir-gonadotropes in the PT and an increase in gonadotropin hormone production [2.14].

Function of the PT is unknown. It has been reported that the PT is rich in melatonin receptors, and there is suggestive evidence that these receptors are found in PT-specific cells [2.15]. In some species, the morphology of PT-specific cells varied with photoperiod and not with functional changes in endocrine organs that are targets of the pituitary [for review refer to 2.16].

Neurohypophysis (Neural Lobe/Pars Nervosa)

The neural lobe is primarily composed of glial cells, pituicytes, axons, nerve terminals, and capillaries [2.2]. Ultrastructural studies in rats [2.17], mice [2.18], rabbits [2.19], and humans [2.20, 2.21] have revealed that there is a striking similarity in anatomy and composition of the neural lobe in various mammals [2.22].

The funnel shaped area of the neurohypophyseal portion of the hypophysis is located at the base of the third ventricle and is referred to as the infundibulum. The principal elements of the infundibulum include axons of magnocellular hypothalamic neurons, axons and terminals of parvicellular hypothalamic neurons which are associated with median eminence vasculature, and glial cells [2.2]. The external portion of the infundibulum principally contains axon terminals and axons of parvicellular neurons, median eminence vasculature, and glial cells. Axons passing through the infundibulum originate from magnocellular perikarya localized in the supraoptic and paraventricular nuclei of the hypothalamus. These axons form the "supraopticohypophyseal tract". Distal segments of these axons are characterized by the presence of large acidophilic non-

terminal dilations, which are filled with neurosecretory substance. These swellings are referred to as Herring bodies [2.2]. Penetration of primary portal plexus capillaries into the central region of the infundibulum creates the neurovascular structure that is closely related to axon terminals of parvicellular hypothalamic neurons. This structure is commonly referred to as the median eminence. Most of the parvicellular axon terminals that are found in this area contain and release various hormones or amines that control hormone secretion from the PD [2.1, 2.4].

The pars nervosa accounts for the largest mass of the equine neurohypophysis (Figure 2.1). It principally contains pituicytes, Herring bodies, axon terminals of magnocellular neurons, and a well developed capillary system. A detailed histological description of the neurohypophysis is not included in this review since structure of this subdivision was not characterized as part of this project.

Blood Supply to the AP

The internal carotid arteries give rise to rostroventral infundibular (hypophyseal) arteries, which supply the ventral region of the primary capillary plexus in the median eminence. The caudal communicating branches of the circle of Willis branch into the rostrodorsal infundibular arteries which supply the dorsal region of the pituitary portal plexus. Median eminence vasculature converges to form the long portal vessels in the external layer of the infundibulum. These vessels break up into a second capillary bed in the PD [2.23-2.26].

The primary function of median eminence capillaries and the pituitary portal vessels is to efficiently carry hypothalamic regulatory compounds to the AP. The second

capillary bed in the AP also facilitates diffusion of pituitary hormones into the systemic circulation for efficient delivery to peripheral target tissues [2.1, 2.4].

Control of LH and FSH Secretion

Gonadotropin-Releasing Hormone (GnRH) Secretion and Effectiveness. Regulation of pituitary LH and FSH synthesis and secretion is primarily controlled by GnRH. Characteristics of gonadotropin secretion are dependent on the pattern of GnRH secretion. Hourly high frequency administration of GnRH to pituitary stalk-sectioned OVX sheep and monkeys preferentially causes the release of LH, whereas, slow frequency GnRH pulses (one injection every 3 hours) resulted in an increase in circulating FSH [2.27, 2.28]. Similar results have been reported for mares during the anestrus season. Specifically, infusion of GnRH every 45 minutes resulted in preferential release of LH whereas GnRH infusion every 6 hours resulted in an increase in circulating FSH [2.29]. Chronic administration of GnRH agonist resulted in down-regulation of LH and FSH secretion which subsequently suppressed an array of reproductive functions [2.30, 2.31]. Chronic infusion (28 days) of GnRH agonist to mares resulted in suppression of LH output by the pituitary [2.32]. Montovan et al. [2.33] reported that mares required up to 30 days of GnRH treatment for any significant suppression to occur; however, chronic treatment with GnRH did not alter the sexual behavior of stallions. Administration of a single large dose of a GnRH agonist to mares prolonged the estrous cycle [2.34]. Thus, horses require prolonged treatment with GnRH to suppress ovarian and sexual functions and equine pituitary GnRH receptors are apparently relatively insensitive to long-term treatment with GnRH.

During estrus, GnRH pulse rate increased to a maximum frequency of two pulses per hour as measured in pituitary venous blood of horse mares [2.35, 2.36]. At the time of maximal GnRH pulse frequency, LH rises and FSH is suppressed. Conversely, in the mid-luteal phase, gonadotropin pulses occur approximately twice daily. Preliminary measurements of GnRH in pituitary venous blood show that GnRH pulse rate is lower during the luteal phase than during the periovulatory surge; however, the decline in GnRH pulse frequency, and a large percentage of GnRH pulses are not effective in stimulating gonadotropin release [2.37]. Whether the apparent uncoupling of GnRH and gonadotropin secretion is a real phenomenon is not clear. It is possible that methods which depend on measurement of GnRH in blood which has already passed through the pituitary gland, and is therefore depleted of decapeptide due to binding of hormone to target cells and enzymatic breakdown, may not detect some secretory pulses of GnRH [2.37].

In horses, secretion of both LH and FSH is normally stimulated by GnRH [2.35, 2.37-2.39]. However, active immunization of intact mares with GnRH-conjugates resulted in a greater reduction in circulating concentrations of LH than FSH [2.40]. The authors concluded that LH and to some degree FSH secretion, and probably LH production, in the mare are dependent upon the bioavailability and quantity of GnRH. However, the lack of effect of GnRH-immunization on FSH response to a GnRH analog suggests that FSH stores in the pituitary may be relatively independent of GnRH bioavailability.

Estradiol Effects on Gonadotropins. Administration of estradiol to women or female animals is followed by an initial suppression of plasma LH and FSH, but both gonadotropins, particularly LH, are subsequently increased [2.41]. These observations have been confirmed in numerous studies and estradiol consistently exerts a biphasic effect on gonadotropin secretion. The time course of the biphasic action depends upon the species and upon the dose of estradiol used [for reviews refer to 2.42, 2.43].

The negative effect of estradiol on pulsatile LH secretion was first observed in ovariectomized monkeys by Yamajia et al. [2.44] in 1972. Infusion of a dose of estradiol, which restored the level of circulating steroid found in the early to mid-follicular phase of the menstrual cycle, led to a profound suppression of the hourly discharge of LH pulses [2.45 2.46]. This negative effect of estradiol plays a major role in the regulation of tonic LH secretion during the course of the menstrual cycle in the rhesus monkey [2.47]. Subsequent studies led to the conclusion that the negative feedback effects of estradiol in the monkey are produced, in large measure, by action on the pituitary which suppresses the response to GnRH [2.42]. The negative effects of estradiol have been studied in other species (for reviews refer to 2.43, 2.48]. Available evidence from these animals indicates that estradiol acts principally on the central nervous system (CNS) in pigs, and exerts significant actions on both the pituitary and the CNS in sheep and cattle.

Estradiol can also exert a positive effect on the pituitary release of LH. Following the administration of estradiol, the pattern of LH secretion varies with time and the extent to which circulating estradiol is elevated. In most mammalian species, if the estradiol

level is high enough (i.e., upper physiological range), the initial negative feedback response is interrupted by an LH surge comparable to the massive discharge that induces ovulation during the estrous cycle [for reviews refer to 2.48- 2.52]. The latency to onset of this response, referred to as the positive feedback response, depends upon the blood level of estradiol achieved. Within limits, the higher the estradiol level, the shorter the latency to the LH surge [2.48, 2.51].

In the mare, endogenous concentrations of estradiol begin to increase 6 to 8 days before ovulation (approximately at the start of the estrus) and plasma estradiol concentrations peak about 2 days before ovulation [2.53, 2.54]. Low luteal-phase values of estrogens are regained within a day or two after ovulation [2.53, 2.54]. In the mare, estradiol administration does not lead to an ovulatory LH surge nor does it cause marked LH suppression. However, long-term treatment of OVX mares with estradiol raises LH levels for the duration of treatment in both the breeding and nonbreeding seasons [2.55, 2.56].

There is some evidence that estradiol may interact with GnRH to influence the form of circulating LH in mare. Treatment of anestrus mares with estradiol followed by hourly pulses of GnRH results in a sustained increase in the relative in vitro biological activity of plasma LH [2.57]. Treatment with either hormone alone does not alter LH bioactivity in seasonally anestrus mares [2.57]. In subsequent studies, the combination of estradiol and GnRH treatments was designed to mimic hormonal events during the periovulatory period when the relative bioactivity of plasma LH normally increases

[2.58]. Results form this work suggest that the interaction between estradiol and GnRH may produce this normal increase in bioactivity of LH.

Effect of estradiol on FSH has not been investigated as thoroughly as effects on LH. However, it is generally agreed that administration of estradiol suppresses FSH in women, monkeys, and sheep [for review refer to 2.59]. Experiments using pituitary cells in culture have shown that estradiol inhibition of FSH responses to GnRH involves inhibition of FSH synthesis. Synthesis of FSH is decreased after 6 to 8 hours of gonadotrope exposure to estradiol [2.54]. Furthermore, immunization of sheep against estradiol raises FSH and increases follicular development [2.59]. In OVX mares, contradictory results have been obtained with estradiol treatment. Some workers have found no effect of estradiol on FSH either in the breeding season or nonbreeding season [2.55], whereas others have reported that FSH was suppressed by estradiol treatment [2.56, 2.60].

Androgen Effects on Gonadotropins. It is well established that androgens regulate the secretion and synthesis of gonadotropins differentially. Testosterone treatment of adult castrated rats results in an increase in pituitary content of FSH, but a decline in LH content [2.61]. More recent studies have shown that differential regulation of in vivo gonadotropin synthesis occurs at the pre-translational level in both male and female rats [2.62-2.65]. In vivo studies have also shown that androgens negatively regulate only α and LHβ subunit mRNA levels, having little or no effect on FSHβ subunit levels [2.62-2.64]. Gharib et al. [2.65] showed that in vitro treatment of rat pituitary cells with androgens positively regulated FSHβ subunit mRNA levels but

has no effect on α or LHβ subunit mRNA levels. However, organ cultures of adult female pituitaries, when treated with DHT for 5 days, have increased stores of FSH but markedly decreased stores of LH [2.66, 2.67]. The effects of DHT on LH and FSH also reportedly include a reduction in LH and FSH secretion, increased pituitary content of FSH, and reduced pituitary content of LH [2.56, 2.68-2.71].

Thompson et al. [2.72] reported that treatment of mares with testosterone propionate (TP) during late estrus or early diestrous reduced circulating FSH concentrations. However, GnRH treatment of these mares resulted in increased plasma FSH levels. Administration of TP during estrus did not have any effect on its duration; however, a postovulatory surge in FSH was measured in these mares [2.72]. Thus, TP treatment of late estrus and early diestrus mares causes an initial suppression and a later rebound in FSH release. Based on these findings, Thompson et al. [2.73] hypothesized that synchronization of mares with altrenogest (a progestogen) could be followed by testosterone to induce a midcycle-like surge of FSH. Related experimental results confirmed that the combined treatment initially decreased plasma FSH levels, but plasma FSH subsequently increased over the pretreatment baseline. Administration of androgens to mares also increased pituitary FSH. This pool of FSH was readily released by GnRH treatment [2.56, 2.73]. Thus, Thompson and coworkers concluded that androgens may play a role in accumulation of pituitary FSH during the estrus period so that a releasable pool of this gonadotropin is available for later release during diestrus.

Other studies by Thompson's group [2.60, 2.71, 2.74-2.76] addressed the question of whether testosterone or aromatized testosterone metabolites are responsible

for TP effects on FSH. In these experiments, ponies were actively immunized against estradiol and later treated with TP. This treatment protocol resulted in about a 50% reduction in the stimulatory effect of TP treatment on pituitary FSH concentrations [2.74]. Thus, androgens and estrogens act together to produce effects of TP on pituitary FSH. Collectively, these data suggested that androgens and androgen metabolites produced by follicles during early diestrus exert a positive effect on FSH. This effect could also be responsible for the diestrus surge of FSH.

Control of GH Secretion

Little is known about GH secretion in horse. However, GH from the equine anterior pituitary gland has been characterized [2.77-2.81]. The amino acid sequence for equine GH [2.82] and nucleotide sequence cDNA for equine GH have been determined [2.83].

Fluctuations in the circulating concentrations of GH in different farm and laboratory animals, primates, and humans have been characterized [2.84-2.89]. Growth hormone secretion is pulsatile or episodic with high amplitude GH pulses superimposed over a low constant baseline in monkeys [2.87] and pigs [2.88] or over a variable irregular baseline in cattle [2.84] and sheep [2.89]. Elevated plasma GH concentrations are not necessarily correlated with any immediate physiological changes [for review refer to 2.86]. However, several physical and metabolic factors have been shown to be related to increased plasma GH concentrations. Factors effecting GH secretion include slow-wave sleep and posture in sheep [2.89]; age (youth) in sheep, monkeys, and pigs [2.85, 2.87, 2.88]; gender in pigs [2.90]; fasting, exercise, trauma, and deep sleep in humans

[for review refer to 2.86]; and poor nutritional status in sheep and cattle [2.91-2.93]. In addition, GH-releasing hormone (GHRH) has been characterized for several species [2.94-2.96]. This hypothalamic hormone results in rapid secretion of GH whereas somatostatin inhibits GHRH-induced GH release and GH synthesis [2.94-2.96].

Stewart [2.97] reported GH release in the horse is pulsatile and similar to secretory patterns reported in other species. Thompson et al. [2.98] reported that although GH concentrations varied in a pulsatile manner in all horses studied, there was no effect of sex or season on plasma GH concentrations and no indication of a diurnal pattern of GH secretion. Treatment of horses with GHRH increased plasma GH concentrations. Plasma GH was also increased when stallions were exposed to estrous mares or following an acute exercise bout. Restraint and/or epinephrine administration also increased plasma GH concentrations. Thompson and coworkers concluded that the secretory patterns of GH in mares and stallions are episodic and/or pulsatile in nature; GH secretion is part of the overall hormonal response to stimulation of the sympathetic nervous system; and there is no co-secretion of GH and prolactin following thyrotropin-releasing hormone (TRH) or sulpiride treatments. In a follow up study, Thompson et al. [2.99] reported GH pulses were more frequent and had a greater amplitude in stallions and geldings as compared to GH secretory patterns in mares. Growth hormone secretion following restraint via a twitch or epinephrine administration was also greater in stallions than in mares or in geldings. Finally, exercise-induced plasma concentrations of GH were greater in geldings than in mares and stallions.

Control of PRL Secretion

Chen and Meites [2.100] and Cheng and Johnson [2.101] reported the stimulatory effects of gonadal steroids on PRL in OVX rats more than twenty years ago. Moreover, there are several reports concerning PRL secretion and storage in horses [2.56, 2.102-2.108]. Johnson [2.105] found no change in PRL secretion throughout the estrous cycle of mares. Worthy et al. [2.104] reported brief increases in PRL secretion at the end of diestrous in three pony mares; however, the exact pattern of PRL secretion during the estrous cycle was not clear. Thompson et al. [2.56] reported that treatment of OVX pony mares with estradiol increased pituitary content of PRL, but this treatment did not increase serum PRL concentrations. These authors also reported that treatment of OVX pony mares with DHT or testosterone increased the PRL response to exogenous TRH, even though neither androgen had any effect on daily secretion or storage of PRL [2.56].

Experiments Testing for the Effects of Androgens and Estrogens on LH, FSH, PRL, and GH

Numerous publications describe the effects of reproductive steroids on production, secretion, and storage of LH and FSH in the equine species [2.55, 2.60, 2.68-2.70, 2.72-2.76, 2.109-2.115]. It is also well established that LH and FSH content of the pituitary is profoundly influenced by reproductive steroids [2.56, 2.75, 2.115]. Thompson and coworkers [2.56] previously evaluated the relationship between hormone secretion and storage and pituitary response to secretagogue after steroid treatment of OVX pony mares. These authors concluded that estradiol had a positive

effect on LH secretion and storage whereas it had a negative effect on FSH secretion. However, estradiol increased the pituitary concentration of FSH. Androgen treatment of the ponies lowered LH secretion and storage and it reduced FSH secretion while increasing storage of FSH.

Hassan et al. [2.116] studied the effect of sex steroids on basal GH and GHRH-stimulated release of GH in bovine pituitary cell cultures. They found that at the doses tested, sex steroids did not affect basal release of GH, but androgens increased GHRH-induced GH release. Studies in rats [2.117, 2.118] showed that pretreatment of castrated males with testosterone significantly enhanced the pituitary GH response to GHRH, whereas pretreatment of OVX female rats with estradiol did not alter the response. Administration of testosterone to juvenile male rhesus monkeys [2.119] increased blood PRL, pituitary PRL content, and the number of lactotropes. However, in baboons [2.120], administration of a physiological dose of estrogen did not increase GH concentrations, whereas a pharmacological dose of estrogen given to intact female baboons increased serum GH. Estradiol treatment of pituitary cultures has yielded both positive and negative results on GH synthesis [2.121-22.4].

Treatment of OVX pony mares with androgens and a subsequent challenge by TRH increased plasma PRL concentrations [2.56]. Apparently androgens enhanced the sensitivity of the pituitary PRL response to TRH. Nogami [2.125] reported that testosterone administration decreased PRL secretion in male rats whereas androgen treatment of juvenile monkeys increased pituitary PRL content and the number of PRL secreting cells [2.119].

Immunocytochemical Staining of Gonadotropes

Prior to the development of immunocytochemical methods, cytologists differentiated two morphological subsets of cells which were believed to synthesize and store gonadotropins [2.126, 2.127]. The classical Kurosumi-Oota [2.127] FSH cell was large, ovoid, and contained a population of large (≥400 nm) and small (~200 nm) diameter granules. The Kurosumi-Oota LH cell was angular and contained scattered granules 200 nm in diameter.

The first electron microscopic-immunocytochemical studies of Nakane [2.128] demonstrated that LH was in the "classical FSH cell" previously described by Kurosumi and Oota [2.127]. Follicle-stimulating hormone was also in the Kurosumi-Oota FSH cell and in a cell which was angular and contained small, peripherally arranged granules. Tougard et al. [2.129] and Tixier-Vidal et al. [2.130] reported the presence of LH and its subunits in both classical FSH and LH cells in male rats. Thus, it was concluded that both gonadotropins can exist in one cell and that one could not distinguish FSH and LH cells on the basis of morphology.

Several investigators have since documented the secretion or localization of more than one hormone by or in individual pituitary cells in different species including dogs [2.131], mice [2.132], rats [2.133, 2.134], pigs [2.135, 2.136], humans [2.137], and sheep [2.138]. However, Basting et al. [2.139] reported that dual-localization of both ir-gonadotropins in one cell type does not hold true for the bovine species.

The introduction of post-embedding immunocytochemistry methods based on specific primary antibodies and immunogold secondary conjugates, especially protein A-gold and gold conjugates to secondary antibodies [2.140-2.142], has enabled

researchers to study the ultrastructural localization of tissue antigens. These techniques facilitate studies on the mechanism of production, transport, and processing of ir-hormones and they help to conclusively demonstrate co-existence of two or more distinct hormones in the same secretory granules [2.143]. This technique also allows one to perform double labeling for ultrastructural immunocytochemical staining of conventionally processed, resin-embedded tissues [2.143].

Gonadotropes comprised 10 to 15% of sheep pituitary cells and, in this cell group, 57% of the cells stained for LH and FSH and about 34% and 8% stained only for LH and FSH, respectively. However, in OVX ewes, 75% of the gonadotropes contained both ir-hormones, 22% of the cells stained only for LH, and 3% stained only for FSH [2.119]. In OVX rats, the gonadotrope cell population included a large (75-85%) cell group that stained for LH and FSH and about 20% of the gonadotropes stained for a single gonadotropin [2.138]. In pigs, 21 to 25% and 24 to 37% of the gonadotropes contained both ir-hormones in intact and OVX animals, respectively [2.136].

Immunocytochemical Staining of Lactotropes and Somatotropes

Immunoperoxidase and immunogold labeling techniques have been used in conjunction with specific primary antibodies to identify cells that produce GH and PRL in normal tissue and in pituitary tumors in monkeys [2.144], humans [2.145-2.148], rats [2.149-2.152], hamsters [2.153, 2.154], pigs [2.155], goats [2.156], cows [2.157-2.160], sheep [2.161-2.163], and musk shrews [2.164-2.166]. Tashjian and coworkers [2.167] suggested that the production of both GH and PRL from one cell type occurs in rodent pituitary tumors. Further studies by Zimmerman et al. [2.168] showed that certain areas

of normal human pituitaries contain numerous cells that stain for both GH and PRL and these cells represented more than 50% of the total number of cells in the AP. They concluded that a significant population of cells normally contain both of these hormones in human pituitaries. More recent investigations, utilizing immunocytochemical techniques and electron microscopic study, have supported the conclusion that PRL and GH normally co-exist in cells of adult and fetal human pituitaries [2.169-2.171], human pituitary adenomas [2.147, 2.168, 2.172-2.175], and normal rat [2.176, 2.177] and cow [2.178, 2.179] pituitaries. Moreover, pituitary cells that secrete both GH and PRL simultaneously (mammosomatotropes) have been reported in studies where hemolytic plaque assay technique was utilized [2.178-2.180].

Several reports have classified lactotropes into two or three types based on morphology of the secretory granules and size of the cells [2.159, 2.160]. These subsets of lactotropes have not always been differentiated in immunocytochemical experiments on pituitary tissues obtained from various domestic species. An electron microscopy study done by Mikami [2.157] indicated that large lactotropes in the bovine pituitary gland were round or oval in shape; these cells contained large, dense granules, which were spherical or polymorphic in shape. Ultrastructural studies by Heath [2.181] indicated that secretory granules of lactotropes in bovine pituitary are the largest in diameter compared to granules in other cells. Results from a similar immunohistochemical study of Dacheux and Dubois [2.158] showed that PRL secreting cells in bovine pituitaries contain large secretory granules. However, these authors emphasized that the granules in lactotropes were smaller in diameter than the secretory granules in somatotropes. In goats [2.156], it has been reported that lactotropes contain

the largest secretory granules as compared to size of secretory granules in other hormone secretory cell groups in the pituitary gland.

Storage and secretion of GH and PRL were typically considered to be a function of two distinct pituitary cell types [2.177]. However, there are reports that a third cell type contains and secretes both hormones. Mammosomatotropes have also been found in mice [2.182], rats [2.177, 2.178, 2.183], musk shrews [2.183], and cows [2.159, 2.160, 2.179]. In lactating rats, 26% of cells in the pituitary contained both GH and PRL [2.177]. However, Thorpe et al. [2.162] reported that only 2 cells out of 1800 cells counted contained both GH and PRL in sheep pituitary. In a similar study, Thorpe and Wallis [2.163] cultured sheep pituitary cells and reported that 3% of the cells stained for both GH and PRL. However, by the end of the 96 hour of culture, <0.2% of the cells contained both ir-PRL and ir-GH. The decrease in the mammosomatotropic cell population was presumed to be due to cell culture, which typically depletes or reduces cellular hormone concentrations, and/or to the removal of hypothalamic factors that support normal functions of these cells.

There seems to be two distinct localization patterns for hormone storage in mammosomatotropes found in musk shrews and rats. In one, PRL and GH were packaged in separate granules within the same cell. These were found in pregnant animals. In the other type of storage pattern, both hormones are co-packaged in the same secretory granules [2.183]. Fumagalli and Zanini [2.159] reported the presence of multi-nucleated mammosomatotropes in bovine pituitary gland. These authors reported that GH and PRL are packaged in three different ways; evenly intermixed together in the

same granule, localized in different granules within the same cell, or localized in the same granules but with the individual ir-hormone segregated in different portions of the granule.

Goluboff and Ezrin [2.184] suggested that ovarian steroids could influence the interconversion of somatotrope and lactotrope cells. Placental steroids (presumably estrogen) were also suggested to be involved in reciprocal shifts between these ir-cell types since exogenous administration of estradiol caused radiolabeled somatotropes to take on the morphological characteristics of PRL-producing cells in the rat [2.185]. Boockfor et al. [2.186] utilized a reverse hemolytic plaque assay with pituitary cell cultures from male rats to provide the first direct evidence that estrogens caused a marked increase in the proportion of ir-mammosomatotropes and a concomitant decrease in the relative proportion of cells that stain only for GH. These authors suggested that estradiol may convert cells that release only GH to those that release both GH and PRL. Other studies have shown considerable fluctuation in the number of cells which produce GH, PRL, or both hormones throughout the annual reproductive cycle of bats and musk shrews and during the transition from early pregnancy through the end of lactation in rats [2.183-2.188].

Estradiol effects on somatotropes is a controversial issue and seems to be species specific. In estradiol-treated rat pituitary cultures, Simard [2.122] reported a 2- to 3-fold increase in GH release as well as cellular content. Bethea and Freesh [2.123] and Copeland [2.120] reported that in primates, physiological doses of estradiol do not stimulate GH release. Kineman et al. [2.124], by means of reverse hemolytic plaque

assay on cow pituitary cell cultures, showed that the percentage of all pituitary cells that released PRL was greater in the early luteal phase (day 1-10) than during other phases of the estrous cycle. However, GH-secreting cells remained unchanged throughout the entire estrous cycle. Furthermore, the increase in total PRL secreting cells was attributed to an increase in the number of cells that secreted both GH and PRL, while the number of GH-only cells decreased without a change in PRL-only cell numbers. These authors concluded that there is a reciprocal interconversion of cells that release only GH and mammosomatotrope cells during the estrous cycle. They also suggested that the relationship between ratios of somatotropes, lactotropes, and mammosomatotropes and stage of reproductive cycle indicates that ovarian steroids may regulate this phenomenon. A similar study compared localization of ir-hormones in pituitaries from bulls and steers to determine the role of gonadal steroids on the ratios of GH-only and dual-hormone secreting cells [2.189]. In gonadally intact bulls, approximately 9% of all pituitary cells stained for GH and PRL, whereas 21% stained only for GH, and 45% stained only for PRL. However, a different scenario existed in steers where mammosomatotropes comprised 22% of the pituitary cell population and cells that stained only for GH or only for PRL accounted for 8% and 46% of the total number of pituitary cells, respectively. These results, along with the results from the cow study, indicated that gonadal steroids may have a profound effect on the secretory characteristics and immunocytochemical staining properties of bovine pituitary cells.

Stratmann et al. [2.185] reported that following 21-day treatment of rats with estradiol there was a transformation of somatotropes to lactotropes. Later, Nogami

[2.125] also showed that stimulation of newborn and adult rats with estradiol resulted in an increase in the number of ir-lactotropes. Finally, Frawley et al. [2.190] reported the presence of a distinct cell population in the pituitary that is capable of producing and secreting both GH and PRL concomitantly. Later on, studies from the same laboratory showed that the fluctuation in numbers of lactotropes and somatotropes is influenced by gonadal steroids [2.124, 2.190] and that there is a bi-directional interconversion of these two cell types which depends upon hormonal cues [2.188]. This fluctuation in proportions of pituitary cells has been proposed to be due to transdifferentiation (lose or gain the ability to secrete GH or PRL) [2.191, 2.192] of lactotropes and somatotropes. Although there is no major change in total cell number of PRL and GH cells due to different hormonal milieu, the possibility of cell division among the cells had not been ruled out. This possibility was examined by Kineman et al. [2.192] where bovine pituitary cell cultures were subjected to several stimuli followed by reverse hemolytic plaque assay. The results indicated that the acidophilic subpopulations are capable of transdifferentiation and no significant cell division was involved. However, the authors reported that estradiol was incapable of increasing PRL in their culture system.

There is no information with regard to ultrastructural morphology and relative abundance of any of the ir-hormone cell types in the equine pituitary. There is also lack of information on the behavior of the pituitary cells to different secretagogues and/or inhibitors. Therefore, the following experiments were preformed to characterize the ultrastructure and immunocytochemical staining properties of hormone-producing cell groups in the pituitary of gonadally intact and OVX pony mares and to study the changes

that steroids would induce in morphology and ratios of ir-LH- , FSH-, GH-, and PRL-containing cells. These studies are expected to determine whether various subgroups of cells contain an ir-hormone. Secondly, results from these experiments may help to differentiate individual pituitary cell populations that are sensitive to steroid feedback.

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CHAPTER III

IMMUNOCYTOCHEMICAL LOCALIZATION OF LH AND FSH IN THE EQUINE PITUITARY

Introduction

Prior to the development of immunocytochemical methods, cytologists differentiated two morphological subsets of cells that were believed to synthesize and store gonadotropins [3.1, 3.2]. The classical Kurosumi-Oota [3.2] FSH cell was large, ovoid, and contained a population of large (≥400 nm) and small (≈200 nm) granules. The Kurosumi-Oota LH cell was angular and contained scattered granules 200 nm in diameter.

The first electron microscopic-immunocytochemical studies of Nakane [3.3] demonstrated that LH was in the "classical FSH cell" previously described by Kurosumi and Oota [3.2]. Follicle-stimulating hormone was also in the Kurosumi-Oota FSH cell and in a cell which was angular and contained small, peripherally arranged granules. Tougard et al. [3.4] and Tixier-Vidal et al. [3.5] reported the presence of LH and its subunits in both classical FSH and LH cells in male rats. Thus, it was concluded that both gonadotropins can exist in one cell and that one could not distinguish FSH and LH cells on the basis of morphology. Since then, several investigators have documented the secretion of more than one hormone by individual pituitary cells in different species including dogs [3.6], mice [3.7], rats [3.8, 3. 9], pigs [3.10], humans [3.11], and sheep [3.12]. However, Basting et al. [3.13] reported that this duality in gonadotropin secretion from one cell type does not hold true in cattle.

The introduction of immunogold techniques, especially protein A-gold and IgG-gold conjugates for post-embedding immunocytochemistry [3.14-3.16], has enabled researchers to study the ultrastructural localization of tissue antigens. This technique facilitates studies on the mechanism of production, transport, and processing of hormones and it helps to conclusively demonstrate coexistence of two or more distinct hormones in the same secretory granules [3.17]. This technique also allows one to perform double labeling for ultrastructural immunocytochemistry on conventionally processed, resin-embedded tissues [3.17].

We previously reported light microscopic results for immunocytochemically stained equine pituitary glands [3.18] that enabled us to differentiate the cell types and determine the distribution of different pituitary cells. This technique, however, only provided a coarse localization of hormones and a general distribution of the different cell types. A more discrete and precise labeling method was necessary in order to delineate more clearly the ultrastructural characteristics of the gonadotropes. Thus, the objectives of the present study were to: 1) identify and possibly differentiate gonadotropes more accurately; 2) determine if gonadotropins are contained in a single cell group or in distinct cell populations; and 3) estimate a relative abundance of each type of gonadotrope in the equine pituitary.

Materials and Methods

Pituitary Fixation and Embedding. Three adult pony mares in different reproductive states were used. The mares were kept on pasture and were fed grass hay as needed to maintain good body condition. The mares were euthanized and the

pituitaries were removed within 7-12 min. The pituitaries were rinsed with saline and placed in freshly prepared 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) plus 0.1% glutaraldehyde (Polysciences, Warrington, PA) buffered with 0.1 M sodium cacodylate (pH 7.3; Electron Microscopy Sciences). While in the fixative, the pituitaries were freed of the surrounding capsule and the anterior pituitary was removed from the neural lobe. The pituitary tissues were then cut into approximately 1 mm^3 pieces and fixation continued for two more hours. Following fixation, ten randomly selected pieces from each animal were removed and cut into smaller pieces; these were used for embedding. Tissues were washed several times in 0.1 M sodium cacodylate buffer to remove any residual aldehyde. Tissues were then dehydrated in 50% ethanol (EtOH) for 15 min, 70% EtOH for 20 min, and 90% EtOH for 30 min. Some of the tissue blocks were processed for routine Epon-Araldite electron microscopy as previously described by Poolsawat [3.19]. Tissues used for immunocytochemistry were infiltrated and then embedded with 60% LR Gold (LRG; Electron Microscopy Sciences) monomer : 40% EtOH for 30 min; 70% LRG : 30% EtOH for 30 min; 100% LRG monomer for 2 h; 100% LRG monomer plus 0.1% benzil (initiator) for 2 h; and finally overnight in 100% LRG monomer plus initiator. All procedures were performed at room temperature. The tissues were then embedded in small gelatin or large BEEM® capsules (Electron Microscopy Sciences). The LRG was polymerized under UV light at -15°C for 24 to 36 h. Ultrathin, 80-90 nm light gold-colored sections were cut on a Sorvall MT2-B ultramicrotome and sections were mounted on 400 mesh gold grids.

Presence of any follicles and/or corpora lutea on the ovaries was recorded and a blood sample from each animal was collected at the time of slaughter in a heparinized tube for progesterone analysis [3.20].

Primary Antibody Specificity and Cross-Reactivity. The polyclonal antibodies used were rabbit anti-equine (e) chorionic gonadotropin (eCG; here it will be referred to as anti-LH) [3.21] and rabbit anti-ovine (o) FSH (oFSH) [3.22]. The peptides used in pre-absorption tests for specificity were: bovine (b) LH (USDA-bLH-B-6), human (h) FSH (hFSH; FSH-I-SIAFD-1, Lot # AFP-5720D), porcine (p) prolactin (pPRL; USDA-pPRL-B-1), porcine growth hormone (pGH; USDA-pGH-B-1), bovine thyroid stimulating hormone (bTSH; USDA-bTSH-I-1), and hACTH (human ACTH-I-SIAFD-1, Lot # AFP-2938C). These peptides were obtained from the National Hormone and Pituitary Program (Rockville, MD). Primary antibodies used in this study were previously tested for specific immunocytochemical staining of pituitary hormones in free-floating sections processed for examination at the light microscopic level (3.18). In these experiments, primary antibodies were pre-absorbed with immunoglobulin fractions that cross-reacted with unrelated pituitary hormones, when the hormones were immobilized on a solid support and subsequently processed for immunocytochemical staining with avidin-biotin-peroxidase conjugates. In the present study, primary antibodies pre-absorbed with cross-reactive immunoglobulin fraction were used for post-embedding immunocytochemical labeling and examination of tissues at the electron microscopic level. To this end, 10 μg of each of the aforementioned hormones, in 5 μl of 0.05 M Tris buffer (pH 7.3), were spotted on nitrocellulose paper and allowed to air dry.

They were then exposed to 4% formaldehyde vapor for 30 min and incubated with 100 µl of each primary antibody preparation for 50 hours at 4°C. Ultrathin sections were incubated with absorbed primary antibodies and processed for immunocytochemical staining as described below.

Immunogold labeling. Sections were first incubated for 10 min on a drop of Tris buffer containing 0.02 M glycine to quench any aldehyde left in the tissue. They were then incubated for 30 min on a drop of Tris buffer containing 1% BSA plus 10% normal goat serum (blocking solution) to reduce non-specific binding. The grids were then transferred to a 35 µl drop of the primary antibody (diluted 1:20 in the blocking solution) and they were incubated with diluted primary antibody for 2 h. Next, grids were blotted and rinsed four times on a drop of Tris buffer for 5 min each. Then the sections were blotted and transferred to a drop of the blocking solution for 5 min. They were then transferred to a 35 µl drop of a 20% solution (as recommended by the supplier) of goat anti-rabbit IgG gold conjugate (Electron Microscopy Sciences) and incubated for 50 min. The sizes of the gold particles used were 6 or 10 nm. The sections were then washed in Tris buffer with 2.5 M NaCl for 10 min and rinsed in distilled water. The final step involved incubating the sections on a drop of a 0.5% formaldehyde for 10 min and rinsing with distilled water followed by complete drying.

Double labeling was done following a protocol described previously by Bendayan [3.17]. Briefly, following the above steps for one side of the grid, the grid was turned over and the same procedure was followed except that the primary antibody and the size of the gold particle were changed. The sections were counter-stained with uranyl acetate

and Sato lead before examination with a Philips 410 Electron Microscope. In addition to the cross-reactivity procedures, other controls consisted of omitting the primary antibody step and testing for non-specific binding of pre-absorbed primary antibodies and gold-conjugates to the tissue. Primary antibodies were pre-absorbed with the peptides used in the preceding section.

Pituitary Cell Counts. To assess relative cell frequencies, ten grids per animal were viewed using an electron microscopy. One section per grid was used to count cells and to assess binding of the primary antibodies and gold-conjugates. Only cells sectioned through a nucleus were counted.

Results

Fixation of equine pituitary tissues in 4% formaldehyde with 0.1% glutaraldehyde followed by low temperature embedding in LRG resulted in an acceptable ultrastructural preservation of the organelles and cell membranes (Figure 3.1). For each primary antiserum, gold particles were concentrated over the secretory granules (Figure 3.1 inset).

Specificity of the Antibodies. Only background binding of gold-conjugates was observed when normal goat serum or Tris buffer was used instead of the specific primary antibody (Figure 3.2). Staining was also equivalent to a nearly undetectable background when the primary antibodies were pre-absorbed with the specific antigen and sections were subsequently incubated with gold-conjugated IgG. For each primary antibody, the homologous antigen markedly reduced or completely abolished staining as illustrated in Figures 3.3 and 3.4. Staining with anti-LH and anti-FSH antibodies was not reduced or

Figure 3.1. A cluster of gonadotropes in LH antibody-immunogold (10 nm)-stained equine pituitary tissue embedded in LRG. Nucleus (N). Bar = 1.5 μm. Inset: An enlargement of an area of the cytoplasm shows gold particles on the secretory granules in a gonadotrope demonstrating distinct localization of LH. Bar = 3 μm.

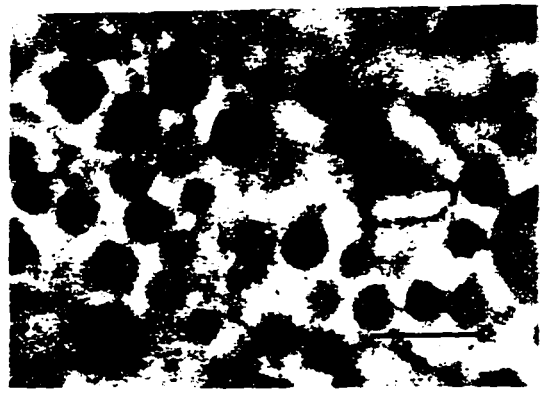

Figure 3.2. Staining and appearance of secretory granules in a gonadotrope processed without primary antibody: Staining is specific for anti-gonadotropin primary antibodies. Bar = .5 μm.

Figure 3.3. Secretory granules in a gonadotrope embedded in LRG and stained with pre-absorbed LH antibody. Binding of gold conjugate was almost abolished when LH antibody was pre-absorbed with the homologous antigen. Bar = .5 μm.

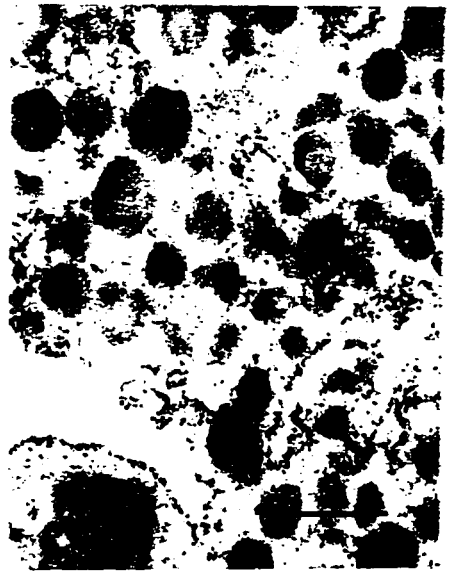

Figure 3.4. Secretory granules in a gonadotrope embedded in LRG and stained with pre-absorbed FSH antibody. Secretory granules in a gonadotrope embedded in LRG. Staining was almost abolished when FSH antibody was pre-absorbed with the homologous antigen. Bar = .5 μm.

abolished when primary antibodies were pre-absorbed with the heterologous gonadotropin (Figures 3.5, 3.6). Results for absorbtion with pGH, bTSH, pPRL, and hACTH are not shown; however, there was no inhibition of binding of primary antibodies or the gold particles to gonadotrope secretory granules when LH and FSH antibodies were absorbed with any of these antigens. Therefore, on pituitary sections, anti-LH antibody reacts only with LH containing cells and not others. Furthermore, the anti-FSH antibody reacts only with FSH containing cells and not others. Some gonadotropes contained vesicles that stained for both LH and FSH (Figures 3.7, 3.8).

Ultrastructural Morphology of the Gonadotropes. Gonadotropes single-stained for LH or FSH were identical in morphology. They appeared as being round or ovoid in shape. The large nuclei tended to be polymorphic and were mostly eccentrically located in the cell (Figures 3.1, 3.9, 3.10). The cells contained scattered and dilated rough endoplasmic reticulum (RER). Gonadotropes were typically large cells but also showed considerable variability in size and ultrastructure. However, gonadotropes could be recognized ultrastructurally by their vesicular RER and the heterogeneous size of their granules (Figures 3.9-3.13). In some cells, the large and dilated cistern of RER had coalesced to form large vacuoles containing material of low electron density (Figures 3.11, 3.12). The mitochondria were usually long and slender, although occasionally spherical, and they were generally concentrated in perinuclear areas.

The secretory granules in gonadotropes could be divided into two types: one was large and polymorphic and was generally located in the middle of the cells with occasional appearance close to the periphery; the other was smaller in size and was

Figure 3.5. Immunoreactive-LH cell processed with LH antibody which was pre-absorbed with FSH. Absorbtion of LH antibody with FSH did not inhibit binding of the primary antibody and (10 nm) gold-conjugate to the LH containing secretory granules. Bar = .5 μm.

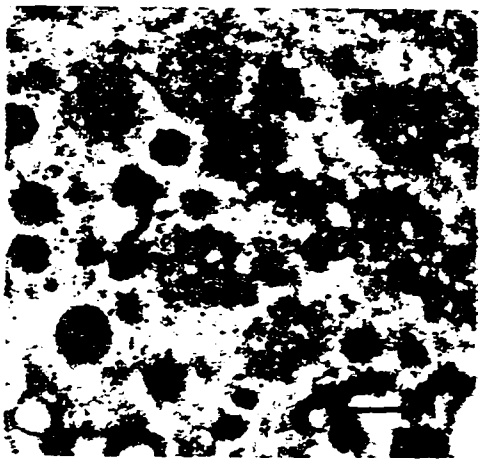

Figure 3.6. Immunoreactive-FSH cell processed with FSH antibody which was pre-absorbed with LH. Absorbtion of FSH antibody with LH did not inhibit binding of the primary antibody and (10 nm) gold-conjugate to the FSH containing secretory granules. Bar = .5 μm.

Figure 3.7. LR Gold-embedded pony pituitary tissue. Cells were double-stained for LH and FSH. LH (10 nm, arrows) and FSH (6 nm, arrow heads). Bar = .7 μm.

Figure 3.8. Double-stained equine pituitary cells processed with LH and FSH antibodies. LH (10 nm, arrows) and FSH (6 nm, arrow head). Bar = .5 μm.

Figure 3.9. Ultrastructure of pituitary cells stained only for FSH. Immunogold (10 nm), single-stained FSH cell. Note the round nucleus (N), large polymorphic granules (small arrow), smaller granules (large arrow) at the periphery of the cytoplasm, and dilated RER (*). The granules in the adjacent cell (AC) are not stained immunocytochemically. Bar = 2 μm.

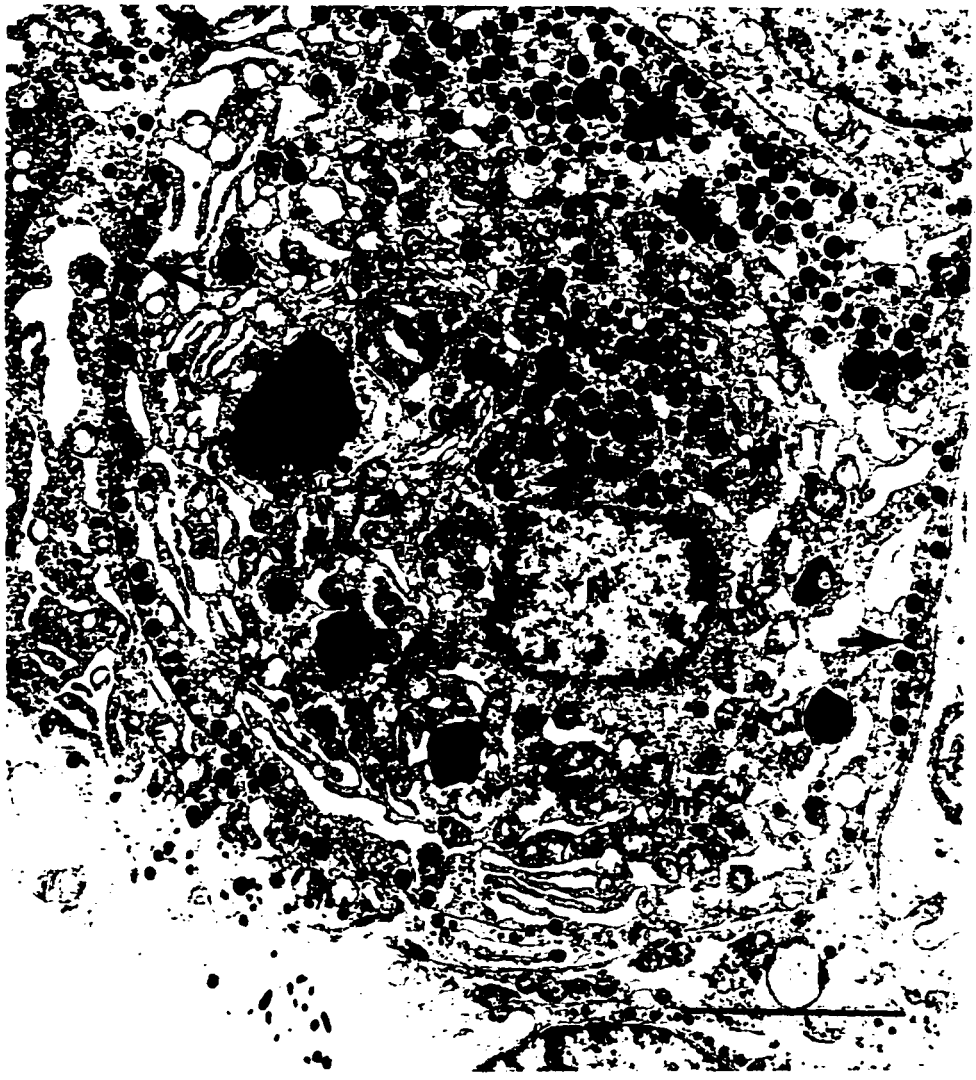

Figure 3.10. Ultrastructure of Epon-embedded gonadotropes in the equine pituitary. Note the dilated RER (*), large polymorphic granules (arrow heads), smaller secretory granules (large arrows) at the periphery of the cytoplasm, mitochondria (m), lysosome (L), Golgi complexes (G), and the nucleus (N). Bar = 3 μm.

Figure 3.11. Immunogold-stained FSH cell in the equine pituitary. Second antibody conjugated to 10 nm gold particles was used to detect specific binding of FSH antibody. Note the vacuolated cytoplasm (*) with the small granules (large arrow) in the periphery and large granules (small arrow) away from the cell membrane. In LRG embedded pituitary tissue, the secretory granules of gonadotropes counter-stained with uranyl acetate and lead appeared less dense than the secretory granules in other cells. The adjacent cell (AC) is a presumptive somatotrope. Bar = 1 μm.

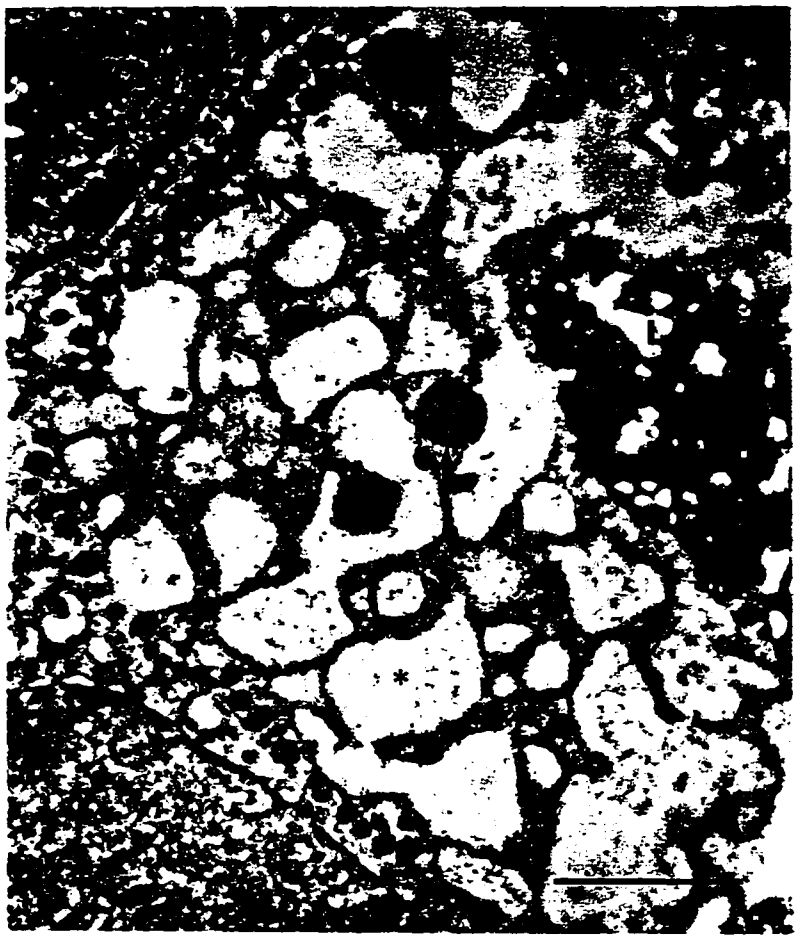

Figure 3.12. Immunogold-stained FSH cell in the equine pituitary. Second antibody conjugated to 10 nm gold particles was used to localize binding of anti-FSH. Note the vacuolated cytoplasm (*) with the small granules (large arrow) in the periphery and large granules (small arrow) away from the cell membrane. The nucleus of an adjacent cell (AC) with its small secretory granules devoid of immunoreactive staining is visible. Bar = 2 μm.

Figure 3.13. Immunogold-stained LH cell in a pony pituitary. Immunogold (10 nm) was used to label bound LH antibody. Note the vacuolated cytoplasm (*) with the small granules (large arrow) in the periphery and the very large polymorphic granules (small arrow) away from the cell membrane. The adjacent cell (AC) with its non-ir granules is visible. Also note that the granules in AC are more electron dense than granules in the gonadotrope. Bar = 2 μm.

generally located along the periphery of the cell (Figures 3.10-3.13). The secretory granules in LRG-embedded gonadotropes appeared less electron dense than the secretory granules in other cell types (Figures 3.11, 3.13). However, electron density was similar among granules in all cell types processed with routine Epon-Araldite fixation (Figure 3.10). In double-labeled sections, some cells stained with many gold particles related to specific-binding for one gonadotropin antibody and a limited number of gold particles related to the second gonadotropin antibody (Figure 3.14).

Cell Numbers and Reproductive Status of the Mares. Based on structures on the ovaries and serum progesterone levels (Table 3.1) it was apparent that the animals were in different stages of the estrous cycle. Pony A was estimated to be in the follicular phase of the estrous cycle. Ponies B and C were estimated to be in the mid- to late luteal and early follicular phase of the cycle, respectively.

Total number of pituitary cells counted per animal using ten grids as well as the percentage of each cell type (LH-only, FSH-only, and cells immunoreactive to both LH and FSH) are presented in Table 3.2. The total population of pituitary cells staining for both LH and FSH ranged from 15 to 32%. For the three mares included in this study, the range for various ir-gonadotropes as a percentage of the total number pituitary cells counted was as follows: LH, 2 to 16%; FSH, 1 to 5%; and LH/FSH, 6 to 24%.

Discussion

Results form the present study show that combining formaldehyde-based tissue fixation with low concentrations of glutaradehyde and subsequent embedding in LRG at low temperature enabled us to preserve the antigenicity of the gonadotropins and the

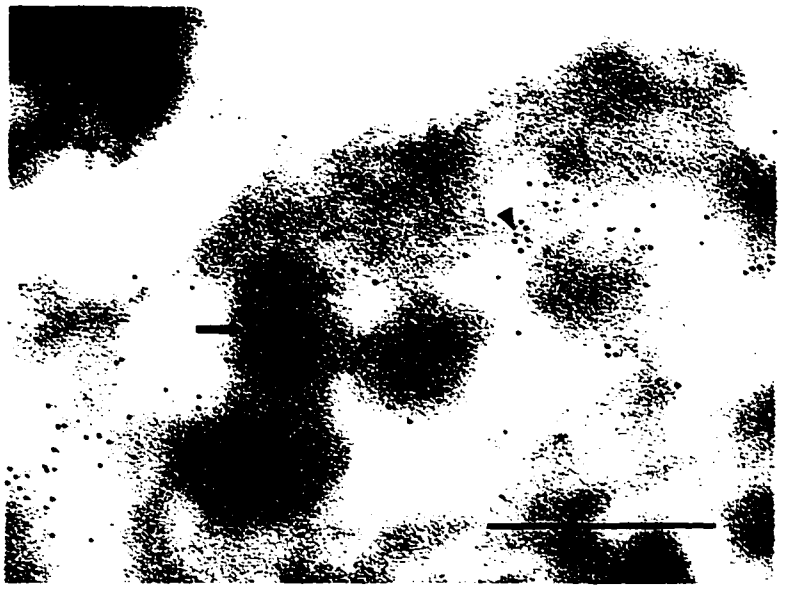

Figure 3.14. Double-stained gonadotrope in a pony pituitary. Note that this cell is predominantly a FSH-containing cell, because most of the granules in this cell are stained for FSH (6 nm, arrow head) whereas a very small amount of LH (10 nm, arrow) is found in these granules. Bar = .3 μm.

Table 3.1. Reproductive status and prominent ovarian structures present at the time of slaughter.

Pony	Slaughter date	Right ovary	Left ovary	Plasma Progesterone (ng/ml)
A	8/3/95	20 mm follicle	40 mm follicle	1.5
B	5/4/94	45 mm follicle 28 x 25 mm CL	40 mm follicle	33.8
C	5/4/94	25 mm follicle	12 mm follicle	2.1

Table 3.2. Relative proportion of the LH and/or FSH containing cells in equine anterior pituitaries.

Pony	Total cells	% LH	% FSH	% Both	% Total
A	178.0	15.7	4.5	6.2	26.0
B	149.0	2.0	1.3	12.0	15.4
C	100.0	7.0	1.0	24.0	32.0
Average	142.3	8.9	2.3	14.0	24.4

Percentages are calculated by dividing the number of ir-cells by the total number of pituitary cells counted in a sample. Only cells with a nucleus were counted.

ultrastructural morphology. Processing of human pituitary with routine Epon embedding techniques allowed Asa et al. [3.23, 3.24] to obtain binding of primary anti-gonadotropin antibodies and secondary gold-conjugates to secretory granules in gonadotropes. Immunocytochemical staining of Epon-embedded equine pituitary samples was attempted but this approach did not allow us to obtain labeling (data not shown). This is probably either due to sensitivity of the LH and FSH molecules to high concentration of glutaraldehyde or the OsO_4 fixation. Dacheux [3.25] previously reported that OsO_4 diminished immunoreactivity of FSHβ subunit but not LHβ subunit in the pig pituitary. The OsO_4 fixation did not abolish antigenic activity of LHβ subunit in bovine [3.26] and ovine pituitary [3.27] but strongly diminished the immunoreactivity of FSHβ subunit. Also, in this study, when gold-conjugated IgG was replaced by gold-conjugated protein A no staining of equine pituitary tissues was obtained.

The present electron microscopic immunocytochemical study on ultrathin sections permitted ultrastructural characterization of gonadotropes within the equine anterior pituitary and provided preliminary data on subsets of cells containing ir-LH and/or FSH. Some cells contained granules labeled for both LH and FSH; this demonstrates that the two hormones may be stored in the same granules and perhaps released simultaneously from a subset of equine gonadotropes. This study has also shown, based on visual observation, that the concentrations of ir-FSH and LH can vary from cell to cell, and that some cells contain only FSH or LH. These cells represent two additional subsets of equine gonadotropes. However, the possibility cannot be excluded that these cells do

contain quantities of co-localized LH or FSH that can not be detected with immunocytochemistry.

These results indicate, for the first time, that gonadotropes in the equine pituitary can be divided into FSH-only, LH-only, and cells containing both ir-LH and FSH. Our results are in agreement with results from light and electron microscopic studies of other mammalian species [3.6-3.12]. Although Basting et al. [3.13] reported that the bovine anterior pituitary lacks the cell type that stores both LH and FSH simultaneously, we have processed pituitary tissue from steers in the same manner (data not shown) as our equine pituitaries, and found LH and FSH co-localized in many cells just as in the equine pituitary. The reason for the discrepancy between their results and ours is not clear, but it could be due to the use of different reagents and embedding resin in the two studies.

Plasma progesterone concentrations and ovarian structures at the time of slaughter suggest that these mares were in different stages of the cycle (i.e., under different hormonal milieu). If the cell counts obtained from our three ponies are indeed representative of mares in the various stages of the estrous cycle, we would speculate that the relative abundance of the subsets of equine gonadotropes may be influenced by the hormonal milieu of the animal. Whether or not this possibility is applicable to the equine species will obviously require experiments with more mares in defined stages of the estrous cycle. However, these experiments are justified since progesterone has been shown to inhibit the synthesis of LH secretion [3.22] and it is possible that progesterone also plays a role in regulating the LH-producing cell populations in pony mares (Table 3.2). A possible role for estradiol in regulation of gonadotrope subset can not be ruled

out by this experiment. It is known that estradiol has a positive effect on LH synthesis and secretion in equine pituitary [3.22]. This could contribute to the higher percentage of ir-LH cells in pony A and C. However, a light microscopic study in sheep [3.12] indicated that the relative percentages of gonadotropes were not influenced by the stage of the estrous cycle.

Gonadotropes comprised 10 to 15% of sheep pituitary cells and this population included a subset of cells that stained for both gonadotropins (57% of gonadotropes) and about 34% and 8% of the gonadotropes stained only for LH or FSH, respectively [3.12]. In OVX ewes, 75% of the gonadotropes stained for both hormones, 22% stained only for LH, and 3% stained only for FSH [3.12]. In OVX rats, 75-85% of the gonadotropes stained for LH and FSH and about 20% of the cells stained for only one gonadotropin [3.8]. In pigs, 21 to 25% and 24 to 37% of the gonadotropes contained both ir-hormones in intact and OVX animals, respectively [3.25]. In this study, gonadotropes comprised on the average about 24% of the pituitary cells and this cell group was further subdivided into 2 to 16% LH-only cells, 1 to 5% FSH-only cells, and 15 to 32% dual hormone containing cells.

In conclusion, post-embedding immunocytochemistry methods using immunogold labeling and specific LH and FSH primary antibodies has proven to be a practical approach to characterize the distribution of ir-gonadotropins and ultrastructure of subsets of ir-gonadotropes in the equine pituitary. Results from this experiment have also demonstrated that the "one cell-one hormone" concept does not apply to a certain populations of gonadotropes in the horse pituitary. Preliminary data in this experiment

suggest that physiological and endocrinological effects on subclasses of gonadotropes may provide information on pituitary cell groups affected by feedback from the gonads.

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CHAPTER IV

EFFECTS OF ESTRADIOL-17β AND DIHYDROTESTOSTERONE ON GONADOTROPES IN EQUINE PITUITARIES: AN IMMUNOCYTOCHEMICAL STUDY

Introduction

There are numerous reports on the effects of reproductive steroids on production, secretion, and storage of LH and FSH in the equine species [4.1-4.17]. It is also well established that LH and FSH content of the pituitary is profoundly influenced by reproductive steroids [4.9, 4.13, 4.18]. Thompson and coworkers [4.17] previously evaluated the relationship between hormone secretion and storage and pituitary response to secretagogue after steroid treatment of OVX pony mares. These authors concluded that estradiol had a positive effect on LH secretion and storage whereas it had a negative effect on FSH secretion. However, estradiol increased FSH storage in the pituitary gland. Androgen treatment of the ponies lowered LH secretion and storage and reduced FSH secretion while increasing its storage.

Ultrastructural studies of gonadotropes have been reported for dogs [4.19], mice [4.20], rats [4.21-4.23], pigs [4.24], humans [4.25], and sheep [4.26]. These studies have indicated that there are three classes of gonadotropes, including cells that contain and secrete either FSH or LH and cells that contain and simultaneously secrete both LH and FSH. Similar results have been obtained for pituitary tissues of intact pony mares [4.27]. Light microscopic study of sheep pituitaries by Ginal and Nett [4.26] suggested that the number of gonadotropes in the pituitary did not change during the estrous cycle.

Nevertheless, ovarian steroids are known to influence the secretion and storage of the gonadotropins in the horse pituitary gland [4.1-4.4, 4.7, 4.12, 4.13, 4.16]. The question arises whether alterations in gonadotropins may be related to changes in the number of immunoreactive (ir)-gonadotropes or whether there is merely an alteration in cell content of gonadotropins. It is also not known whether all of the three types of gonadotropes in equine pituitaries are regulated similarly by the gonads and/or individual gonadal steroids. It may be that there is a difference in the responsiveness of a gonadotrope subset to various secretagogues and peripheral hormones. The objective of the present study was to determine if the ultrastructure, ir-staining properties, and/or numbers of cells in each subset of the gonadotrope population in OVX pony mares change when animals are subjected to long-term treatment with estradiol or dihydrotestosterone (DHT).

Materials and Methods

Animals and Treatments. Twelve adult (6 to 12 years of age) long term (>2 years) OVX pony mares weighing between 168 and 323 kg were kept on pasture and fed grass hay as needed to maintain good body condition. The mares were randomly assigned to one of three treatment groups (n = 4/group). Group 1 (C) was injected with 1 ml of corn oil. The oil was also used as a vehicle for treatment administration to Groups 2 and 3. Group 2 received 35 µg/kg of bodyweight per day of estradiol (Sigma Chemical Co., St. Louis. MO) and Group 3 received 150 µg/kg of bodyweight per day of DHT (Sigma Chemical Co.). All injections were given subcutaneously in the neck region. Treatment injections were repeated daily at 0800 for 21 days immediately after the daily blood samples were drawn. Due to the time needed for processing tissues for

electron microscopy and RIA, the starting days for injections were staggered over four consecutive days so that pituitaries of only three animals, one from each treatment group, were collected for processing on day 22 when the animals were sacrificed. The injections were started and tissues processed between July 11 and August 4, 1994.

Daily blood samples were collected via jugular venipuncture immediately prior to the daily injections. These samples were drawn into heparinized tubes; plasma was harvested by centrifugation, and samples stored at -15°C for later analysis.

Pituitary Preparation for Hormone Analysis. The pituitaries were removed within 7 to 12 min following euthanasia by electrocution. Tissues were rinsed with saline and cut mid-sagittaly. One half of each pituitary was placed in ice-cold .01 M phosphate buffered saline (.15 M NaCl; PBS). Tissues were stripped of the surrounding capsule, the neural lobe was separated from the anterior pituitary (AP), and the halved AP was weighed. This AP sample was homogenized in 100 ml of PBS in a Waring 7011 blender (Waring Prod. Div., Dynamics Corp. of America, New Hartford, CT) at maximum setting for 30 sec. Another 100 ml of PBS was used to rinse out the blender. The homogenates were mixed well and 10 ml aliquots were centrifuged at 1200 x g for 30 min at 4°C. The supernatants were harvested and frozen at -20°C. Pituitary homogenates were diluted 1:500 in PBS containing 0.1% (w/v) gelatin. Concentrations of LH and FSH in plasma samples and pituitary homogenates were measured by RIA as described previously [4.28, 4.29].

Tissue Preparation for Electron Microscopy. Following a rinse with saline, the other half of AP was placed in freshly prepared 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) plus 0.1% glutaraldehyde (Polysciences, Warrington, PA) buffered with 0.1 M sodium cacodylate (pH 7.3; Electron Microscopy Sciences). While in the fixative, the pituitaries were freed of the surrounding capsule and the AP was removed from the neural lobe. The pituitary tissues were then cut into approximately 1 mm^3 pieces and fixation continued for 2 h. Some of the tissue blocks were processed for routine Epon-Araldite electron microscopy as previously described by Poolsawat [4.30]. Following fixation, ten randomly selected pieces from each animal were removed and cut into smaller pieces; these were used for embedding. Tissues were washed several times in 0.1 M sodium cacodylate buffer to remove any residual aldehyde. Tissues were then dehydrated in 50% ethanol (EtOH) for 15 min, 70% EtOH for 20 min and 90% EtOH for 30 min. Tissues that were going to be immunocytochemically stained were infiltrated and then embedded with 60% LR Gold (LRG; Electron Microscopy Sciences) monomer : 40% EtOH for 30 min; 70% LRG : 30% EtOH for 30 min; 100% LRG monomer for 2 h; 100% LRG monomer plus 0.1% benzil (initiator) for 2 h; and finally overnight in 100% LRG monomer plus initiator. All procedures were performed at room temperature. The tissues were then embedded in small gelatin or large BEEM® capsules (Electron Microscopy Sciences). The LRG was polymerized under UV light at -15°C for 24 to 36 h. Ultrathin, 80-90 nm light gold-colored sections were cut on a Sorvall MT2-B ultramicrotome and sections were mounted on 400 mesh gold grids.

Immunogold Labeling. Sections were first incubated for 10 min on a drop of 0.05 M Tris (pH 7.3) containing 0.02 M glycine to quench any aldehyde left in the tissue. They were then incubated on a drop of Tris buffer containing 1% BSA plus 10% normal goat serum (blocking solution) to reduce non-specific binding. The grids were then transferred to a 35 µl drop of the primary antibody (diluted 1:20 in the blocking solution). Sections were incubated with characterized primary antibodies [4.27] for 2 h. Next, grids were blotted and rinsed four times on a drop of Tris buffer for 5 minutes each. Then the sections were blotted and transferred to a drop of the blocking solution for 5 min. They were then transferred to a 35 µl drop of a 20% solution (as recommended by the supplier) of goat anti-rabbit IgG gold conjugate (Electron Microscopy Sciences) and incubated for 50 min. The size of the gold particles used were 6 or 10 nm. The sections were then washed in Tris buffer with 2.5 M NaCl for 10 min, rinsed in distilled water, and dried. The final step involved incubating the sections on a drop of a 0.5 % formaldehyde for 10 min and rinsing with distilled water followed by complete drying.

Double-labeling was done following a protocol described previously by Bendayan [4.31]. Briefly, following the above steps for one side of the grid, the grid was turned over and the same procedure was followed except that the primary antibody and the size of the gold particle conjugated to the secondary antibody were changed. The sections were counter-stained with uranyl acetate and modified Sato lead before examination with a Philips 410 Electron Microscope. In addition to the cross-reactivity procedures, other controls consisted of omitting the primary antibody step and testing for

immunocytochemical staining of tissues processed with primary antibodies pre-absorbed with various purified pituitary hormone preparations.

Statistical Analysis. Data consisting of repeated measurements taken over time were analyzed by ANOVA that accounted for the repetitive nature of the sampling. Hormonal concentrations in daily samples were calculated as a percentage of the pretreatment concentrations before analysis. The least significant means (LSM) test was used to compare post-treatment means to data for the pretreatment samples. Non-repetitive data were analyzed by one-way ANOVA and means for each treatment were compared to controls by the LSM test. All data were analyzed on PC-SAS version 6.11 [4.32].

Results

Daily Hormone Concentrations. Day 1 pretreatment gonadotropin concentrations (means ± SE) were: Group 1/vehicle-treated, LH = 7.2 ± 1.7 ng/ml, FSH = 65.6 ± 25.3 ng/ml; Group 2/estradiol-treated, LH = 6.4 ± 2.0 ng/ml, FSH = 51.1 ± 30.0 ng/ml; Group 3/DHT-treated, LH = 9.4 ± 5.0 ng/ml, FSH = 70.8 ± 12.5 ng/ml. Plasma LH concentrations, in vehicle- and estradiol-treated ponies did not change ($P > .1$) over time (Figure 4.1a). There was a significant ($P < .001$) difference in gonadotropin concentrations measured in the three treatment groups. Treatment with DHT reduced ($P < .0001$) LH secretion by the end of the treatment period.

Plasma concentrations of FSH in vehicle-treated ponies did not vary ($P > .1$) from pretreatment levels during the twenty one-day period. Treatment with DHT and estradiol reduced ($P < .05$) plasma FSH concentrations (Figure 4.1b).

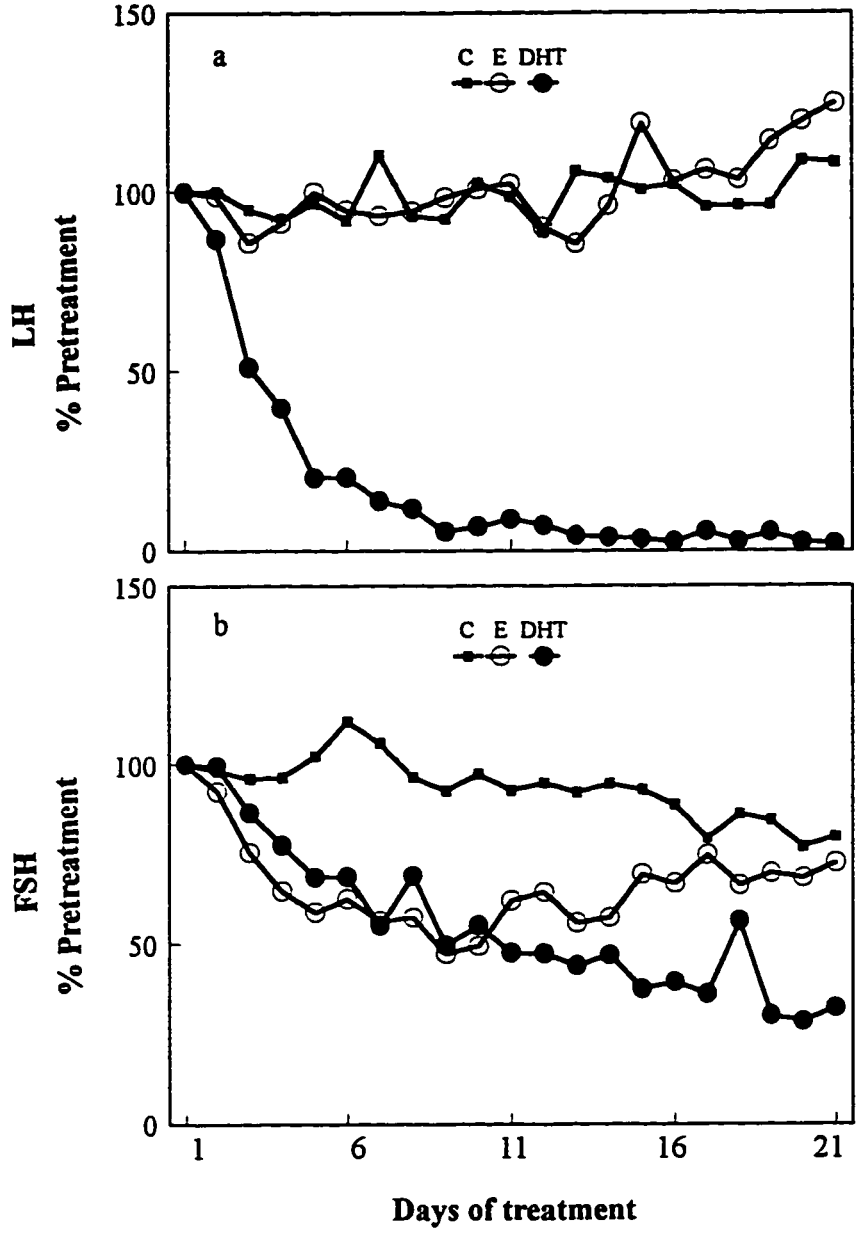

Figure 4.1. Mean daily plasma concentrations of LH (a) and FSH (b) as a percentage of the pretreatment value for mares administered oil, estradiol, or DHT. Mares were administered oil (C), estradiol (E), and DHT daily for 21 days. Data were analyzed by analysis of variance.

Hormone Content in the Pituitary. Pituitary content of LH and FSH in steroid-treated OVX mares are presented in Figure 4.2. One animal in the control group had extremely high values for LH and FSH. Statistical analysis determined that this animal was an outlier ($P < .01$). Therefore, data from the other three mares in the control group were used to determine treatment effects on pituitary content of LH and FSH. Treatment with DHT caused a reduction ($P < .05$) in pituitary LH content. Estradiol treatment significantly ($P < .05$) increased LH content compared to DHT- and vehicle-treated animals. Pituitary content of FSH was not influenced ($P > .1$) by treatment with DHT. However, estradiol caused a significant ($P < .05$) increase in the concentrations of FSH in the pituitaries.

Pituitary Cell Counts. The number of cells which stained only for LH or FSH and the number of cells containing both ir-gonadotropins are listed as a percentage of the total number of cells in Table 4.1. Total number of cells counted for each treatment group were: Oil, 512; estradiol, 657; and DHT, 648. There was no treatment effect ($P > .1$) on the number of cells staining only for LH. However, there was a significant ($P < .05$) treatment effect on cells staining only for FSH and cells containing both LH and FSH. Treatment with DHT reduced ($P < .05$) the number of cells stained for both hormones as compared to double-labeled cells in the estradiol-treated and the control group. Tissues from animals treated with DHT also had more ($P < .05$) cells which stained only for FSH. In all three treatment groups cells containing only ir-LH constituted a very small percentage of the total cell population. Estradiol did not affect ($P > .1$) the population of cells immunoreactive for both gonadotropins when compared

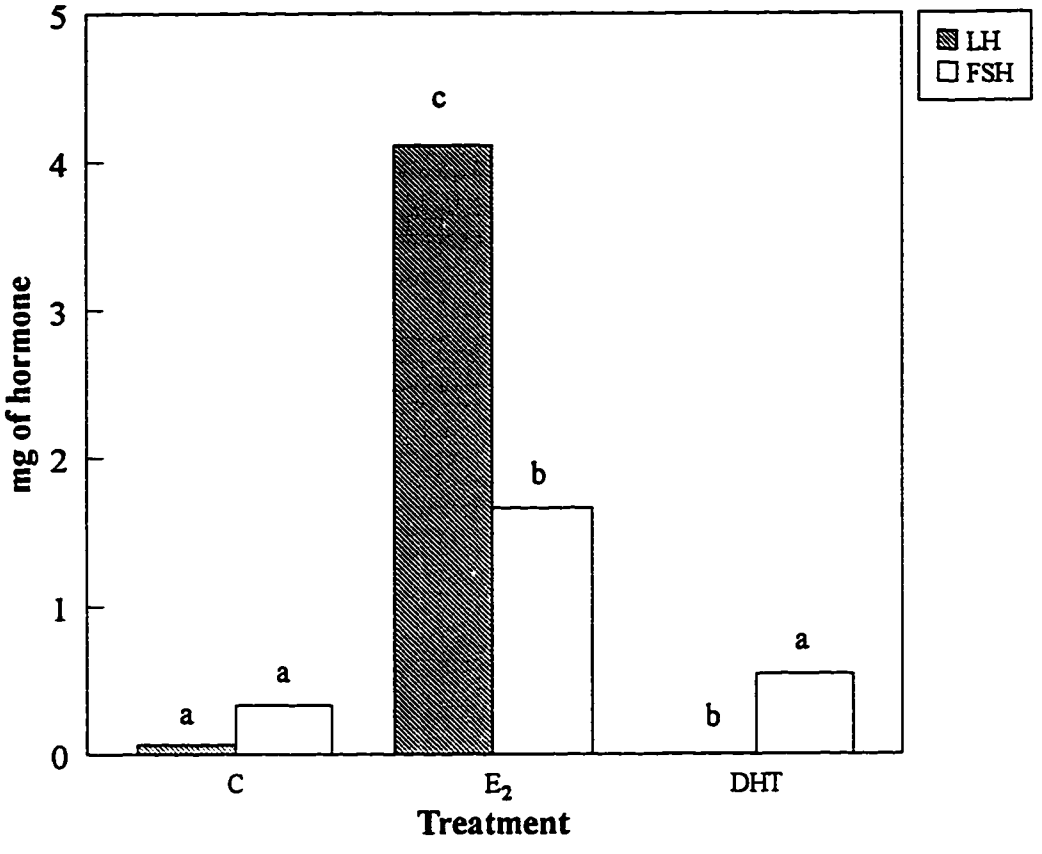

Figure 4.2. Pituitary content of LH and FSH in control and steroid-treated OVX pony mares. Mares were treated with oil (C), estradiol (E), or DHT daily for 21 days. Estradiol significantly ($P < .05$) increased pituitary LH and FSH content compared to C- and DHT-treated mares. a, b, c: Mean with different letters were significantly ($P < .05$) different.

Table 4.1. Mean (M) and relative percentage (%) of ir-LH, ir-FSH and ir-LH/FSH (Both) stained cells in steroid- or vehicle- (Control) treated OVX pony mares.

	Control	Estradiol	DHT
Cell type	**M[a] (%)**	**M[a] (%)**	**M[a] (%)**
LH	1.8 (1.4)[1]	1.3 (0.8)[1]	1.0 (0.6)[1]
FSH	2.3 (1.8)[2]	6.8 (3.2)[2]	14.5 (9.0)[3]
Both	11.5 (9.0)[4]	12.8 (7.8)[4]	5.3 (3.2)[5]
Total	15.5 (12.1)[6]	20.8 (11.7)[6]	22.8 (12.4)[6]

[a]Means represent average number of cells counted per grid (10 grids were counted for each horse).

The SE for mean values of LH, FSH, and Both were 1.0, 2.2, and 2.0, respectively.

[1,2,3,4,5,6]Different superscripts in each row indicate a significant ($P < .05$) difference in mean values within that row.

Percentages are calculated by dividing the number of ir-cells by the total number of pituitary cells counted. Total number of cells counted for each treatment group were: Oil, 512; estradiol, 657; and DHT, 648.

to the control group. The percentage of pituitary cells which were gonadotropes was not influenced ($P > .1$) by different steroid treatments.

Ultrastructural Morphology of Gonadotropes. In Epon-embedded pituitary, gonadotropes from the vehicle-treated OVX pony mares were extremely vacuolated and they contained a small number of secretory granules scattered throughout the cytoplasm (Figure 4.3). Vacuolation of the cytoplasm was also seen in LR Gold-embedded pituitary tissues (Figure 4.4). Mitochondria were generally long and slender and were found in close proximity of each other in the cytoplasm (Figures 4.5, 4.6). The subset of gonadotropes stained for both gonadotropins in the DHT-treated group was predominantly stained for ir-FSH (Figure 4.6).

Biological Activity of Estradiol-17β. Estradiol did not affect LH secretion based on plasma LH and the number of ir-LH-producing cells in the pituitary. Thus, the biological activity of estradiol used for treatment injections was questioned. A uterine bioassay was used to assess bioactivity of the estradiol used in this experiment. Eight immature rats (25 days old) were divided into four groups (2/group) which received a 1 ml injection of corn oil (vehicle) or 100 μg, 10 μg, or 1 μg of estradiol in vehicle. Six hours later the uteri of the animals were excised and weighed. The estradiol used in this experiment caused a significant ($P < .05$) increase in uterine weight and therefore it was biologically active. The results are summarized in Table 4.2.

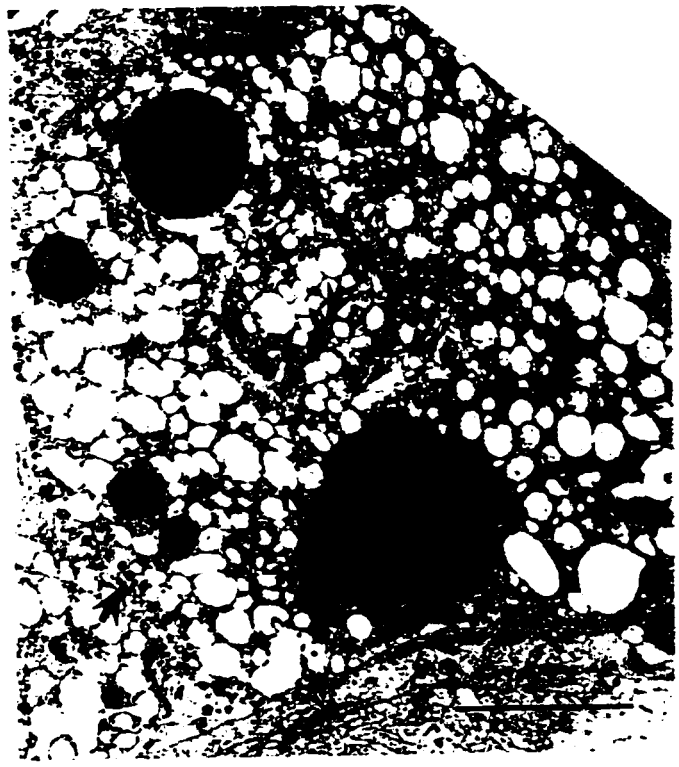

Figure 4.3. Epon-embedded pituitary tissue of an OVX pony mare treated with oil. Note the degree of vacuolization. Small secretory granules (arrows) are scattered throughout the cytoplasm. Lysosomes (L). Nucleus (N). Bar = 5 μm.

Figure 4.4. LR Gold-embedded pituitary tissue of an OVX pony mare treated with oil. The cytoplasm is highly vacuolated (*) with a small number of scattered granules. Adjacent cell is a presumptive somatotrope. Bar = 2 μm. Inset: An enlarged area of the cytoplasm with labeled granules. This cell is labeled primarily for FSH and it contains relatively less immunogold conjugate associated with LH-specific primary antibody (FSH = 6 nm immunogold, arrow head; LH = 10 nm , arrow). Bar = .2 μm.

Figure 4.5. LR Gold-embedded pituitary tissue of an OVX pony mare treated with oil. Note the vacuolization (*) of the cytoplasm along with the low number of secretory granules. Mitochondria (M) are generally long and slender. Nucleus (N). Bar = 1 μm.

Figure 4.6. Characteristic appearance of double-labeled gonadotropes in OVX pony mares treated with DHT. LH = 10 nm, arrows; FSH = 6 nm, arrow heads. Note that the granules are predominantly stained for FSH. Bar = 1 μm.

Table 4.2. Mean uterine weight in rats treated with different amounts of estradiol.

Estradiol	Uterine wt (mg ± SE)
100 µg	$0.11^{1} \pm .020$
10 µg	$0.1^{1} \pm .010$
1 µg	$0.07^{2} \pm .003$
C	$0.07^{2} \pm .002$

[1,2] Different superscripts indicate a significant ($P < .05$) difference.

Discussion

Farquhar and Rinehart [4.33] reported that gonadotropes in gonadectomized rats have a "signet-ring" shape. However, in this study we did not observe any equine gonadotropes with cellular characteristics of signet cells.

There are conflicting reports on the affects of androgens on gonadotropin synthesis and the pituitary concentrations of gonadotropin subunits and the related mRNAs. In vivo studies, in rats, have shown that androgens negatively regulated only α and LHβ subunit mRNA levels, having little or no effect on FSHβ subunit mRNA levels [4.34-4.36]. Gharib et al. [4.37] reported that in vitro treatment of rat pituitary cells with androgens positively regulated FSHβ subunit mRNA levels but has no effect on α or LHβ subunit mRNA levels. In other reports, testosterone treatment of adult castrated rats resulted in an increase in pituitary content of FSH, but a decline in LH content [4.38]. Publications have also claimed that the differential in vivo regulation of gonadotropin synthesis occurs at the transcription level in both male and female rats [4.34- 4.36, 4.39]. Organ cultures of adult female pituitaries, when treated with DHT for 5 days, have increased stores of FSH but markedly decreased stores of LH [4.40]. The effects of DHT on LH and FSH also reportedly include reduced LH and FSH secretion, increased pituitary content of FSH, and reduced pituitary content of LH [4.10, 4.12, 4.13, 4.16, 4.17]. In the present study, plasma LH and FSH concentrations were reduced in DHT-treated animals; this reduction was related to lower pituitary concentration of LH, but the reported positive effect of DHT on FSH was not seen at the pituitary level. The reason for the lack of effect of DHT on pituitary FSH concentration is not clear.

However, based on the above findings, we can probably deduce that DHT treatment of OVX pony mares inhibited LH synthesis or increased LH degradation in ir-LH cells, thereby reducing concentrations and the releasable pool of LH. In support of this possibility, the reduced staining for LH in double-labeled cells suggests that treatment of OVX pony mares with DHT inhibited LH production or increased LH degradation. Furthermore, the reduction in the number of cells staining for both gonadotropes is probably due to an inhibitory effect of DHT on ir-LH in gonadotrope subsets that normally stain for both gonadotropins.

Despite the lack of a DHT affect on FSH content in the pituitary in this study, the number of cells containing FSH increased in DHT treated group. Gonadotropes in DHT-treated animals primarily stained for FSH and very few cells which stained only for LH were counted. Nevertheless, the percentage of pituitary cells that were stained as gonadotropes did not change following DHT treatment. Therefore, we believe that any increase in FSH-only cells is probably due to an inhibition of ir-LH detected by immunocytochemistry and not division of the FSH-only cell numbers. This might also suggest that androgens have a more profound effect on ir-LH than FSH and that they selectively act on the subset of gonadotropes that contains both ir-gonadotropins.

Estradiol increased LH content of the pituitary. However, this increase was neither reflected in the concentrations of estradiol in blood samples or the number of cells stained only for LH. Previous studies have shown that administration of estradiol to OVX mares results in a significant increase in plasma LH concentration and the pituitary content of LH [4.17]. However, estradiol-treated animals in the present study had

decreased plasma FSH concentrations and a moderate increase in the pituitary FSH content. Furthermore, the estradiol used in this study tested positive when used in a rat uterine bioassay. Therefore, there is no doubt that the estradiol given to these animals was biologically active. The reason as to why estradiol did not increase plasma LH in the OVX mares is not clear. However, estradiol may not have elicited a positive response in LH release since these ponies were OVX more than two years prior to the beginning of this study and LH producing cells in the pituitary and/or estrogen-sensitive loci in the brain may have become unresponsive to the stimulatory effects of estradiol at the dosage used. Perhaps due to the long-term ovariectomy, the amount of estrogen used increased LH synthesis or reduced LH degradation without affecting the release of LH or the ir-staining of this gonadotropin.

Exogenous estradiol and DHT were measurable in the plasma samples in the study with OVX pony mares done by Thompson et al. [4.17]. However, levels of these steroids were undetectable in the present study. The estradiol used in this study was suspended in corn oil. Solid vegetable shortening was used by Thompson et al. [4.17] as a vehicle to deliver steroid treatments. There is the possibility that crystalline estradiol and DHT were released at a more gradual rate from the solid shortening whereas the release from the corn oil was more rapid. Thus, estradiol and DHT treatments used in this experiment may not have provided the same duration of increased levels of plasma estradiol and DHT. Another possible reason as to why more ir-LH cells were not detected despite an increase in pituitary LH could be that the newly synthesized LH molecules were not detected by our immunocytochemistry technique. The antigenic

specificity of polyclonal antibody against protein hormones may differ for radioimmunoassay and immunocytochemistry [4.41]. Further studies using techniques such as reverse hemolytic plaque assay and mRNA measurement might help to clarify the discrepancy in steroid affects on pituitary and plasma concentrations of LH.

When unstimulated, the percentage of ir-LH and ir-FSH cells in the pituitary seem to be relatively equal and lower than the number of cells that are immunoreactive for both gonadotropins. Steroid feedback may alter the ratio of cells staining for individual or both gonadotropins without changing the total number of gonadotropes. This suggests that most equine gonadotropes have the capability to store both gonadotropins and that this subset of cells is most sensitive to steroid feedback. The two cell fractions which stained only for LH or FSH may contain low or undetectable levels of the alternate gonadotropin. Nevertheless, the equine pituitary does indeed contain three ir-gonadotrope subsets. Cells that stained for LH-only were not affected by steroid treatment. However, cells that stained for FSH-only and for both LH and FSH were affected by steroid treatment.

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CHAPTER V

IMMUNOCYTOCHEMICAL LOCALIZATION OF PROLACTIN AND GROWTH HORMONE IN THE EQUINE PITUITARY

Introduction

Immunoperoxidase and immunogold labeling techniques have been used to identify cells that produce growth hormone (GH) and prolactin (PRL) in normal tissue and in pituitary tumors in monkeys [5.1], humans [5.2-5.5], rats [5.6-5.9], hamsters [5.10, 5.11], pigs [5.12], goats [5.13], cows [5.14-5.17], sheep [5.18-5.20], and musk shrews [5.21-5.23]. Tashjian and coworkers [5.24] suggested that the production of both GH and PRL from one cell type occurs in rodent pituitary tumors. Zimmerman et al. [5.25] reported that certain areas of normal human pituitaries contain numerous cells that stain for both GH and PRL and these cells represented more than 50% of the total number of cells in the anterior pituitary. These investigators concluded that a significant population of cells normally contain both of these hormones in human pituitaries. More recent investigations utilizing immunocytochemical techniques at the electron microscopic level have supported the conclusion that PRL and GH normally co-exist in cells of adult and fetal human pituitaries [5.26-5.28], human pituitary adenomas [5.5, 5.25, 5.29-5.32], and normal rat [5.33, 5.34] and cow [5.16, 5.17] pituitaries, and are referred to as mammosomatotropes. Moreover, hemolytic plaque assays of cells from normal and tumorous rat and human pituitaries, as well as cow pituitary cells in culture, have shown that mammosomatotropes can secrete PRL and GH simultaneously [5.35-5.37].

We previously reported [5.38] light microscopy results for immunocytochemically stained pituitary glands collected from ponies. That study enabled us to differentiate cell types and to determine the distribution of different pituitary cell types in the equine species. The light microscopic technique, however, only provided a coarse localization of hormones and a general distribution of the cell types that stained for a single hormone. A more precise labeling method was necessary to clearly delineate the ultrastructural characteristics of lactotropes and the somatotropes and to determine whether immunoreactive (ir) hormones are co-localized in pituitary cell populations in the equine species. Thus, the objectives of the present study were to: 1) identify and differentiate lactotropes and somatotropes at the ultrastructural level; 2) determine whether PRL and GH are contained within separate cell populations; 3) determine whether the equine pituitary contains mammosomatotropes; and 4) characterize the relative abundance of the lactotropes, somatotropes, and mammosomatotropes in the equine pituitary.

Materials and Methods

Pituitary Fixation And Embedding. Three adult pony mares were used for this experiment. The mares were kept on pasture and were fed grass hay as needed to maintain good body condition. They were sacrificed by electrocution and the pituitaries were removed within 7-12 min. Presence of any follicles and/or corpus luteum on the ovaries were recorded and a blood sample from each animal was collected in a heparinized tube at the time of slaughter for progesterone analysis [5.39]. Pituitary tissues were rinsed with saline and fixed by immersion in freshly prepared 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) plus 0.1%

glutaraldehyde (Polysciences, Warrington, PA) buffered with 0.1 M sodium cacodylate (pH 7.3; Electron Microscopy Sciences). While in the fixative, the pituitaries were freed of the surrounding capsule and the AP was removed from the neural lobe. The pituitary tissues were then cut into approximately 1 mm^3 pieces and fixation continued for 2 h. Following fixation, ten randomly selected pieces from each animal were removed and cut into smaller pieces; these were used for embedding. Tissues were washed several times in 0.1 M sodium cacodylate buffer to remove any residual aldehyde. Tissues were then dehydrated in 50% ethanol (EtOH) for 15 min, 70% EtOH for 20 min and 90% EtOH for 30 min. Some of the tissue blocks were processed for ultrastructural study using routine Epon-Araldite embedding and transmission electron microscopy as previously described by Poolsawat [5.40]. The tissues processed for immunocytochemical staining were infiltrated with 60% LR Gold (LRG; Electron Microscopy Sciences) monomer : 40% EtOH for 30 min; 70% LRG : 30% EtOH for 30 min; 100% LRG monomer for 2 h; 100% LRG monomer plus 0.1% benzil (initiator) for 2 h; and finally overnight in 100% LRG monomer plus initiator. All procedures were performed at room temperature. The tissues were then embedded in small gelatin or large BEEM® capsules (Electron Microscopy Sciences). The LRG was polymerized under UV light at -15°C for 24 to 36 h. Ultrathin, 80-90 nm light gold-colored sections were cut on a Sorvall MT2-B ultramicrotome and sections were mounted on 400 mesh gold grids.

Primary Antibody Specificity and Cross-Reactivity. The polyclonal primary antibodies used were rabbit anti-porcine (p) PRL [5.41] and rabbit anti-bovine (b) GH which was prepared by the late Dr. Choh Hao Li and colleagues (University of California,

San Francisco) and made available to us by Dr. Harold Papkoff (University of California, Davis). Peptides used to test cross-reactivity of these antibodies included: bLH (USDA-bLH-B-6), human (h) FSH (hFSH-I-SIAFD-1, Lot # AFP-5720D), pPRL (USDA-pPRL-B-1), pGH (USDA-pGH-B-1), bovine thyroid stimulating hormone (bTSH; USDA-bTSH-I-1), and hACTH (human ACTH-I-SIAFD-1, Lot # AFP-2938C). These peptides were obtained from the National Hormone and Pituitary Program (Rockville, MD). Primary antibodies used in this study were previously tested for specific immunocytochemical staining of pituitary hormones in free-floating sections processed for examination at the light microscopic level [5.38]. In those experiments [5.38], primary antibodies were pre-absorbed with immunoglobulin fractions that cross-reacted with unrelated pituitary hormones, and the hormones were immobilized on a solid support and subsequently processed for immunocytochemical staining with avidin-biotin-peroxidase conjugates. In the present study, primary antibodies pre-absorbed of cross-reactive immunoglobulin fraction were used for post-embedding immunocytochemical labeling and examination of tissues at the electron microscopic level. To this end, 10 μg of each hormone, in 5 μl of 0.05 M Tris buffer (pH 7.3), was spotted onto nitrocellulose paper and allowed to air dry. The immobilized peptides were then exposed to 4% formaldehyde vapor for 30 min and incubated with 100 μl of each antibody preparation for 50 hours at 4°C. Ultrathin sections were incubated with absorbed primary antibody preparations and processed for immunocytochemical staining as described below.

Immunogold Labeling. Sections were first incubated for 10 min on a drop of Tris buffer, containing 0.02 M glycine to quench any aldehyde left in the tissue. They were then incubated for 30 min on a drop of Tris buffer containing 1% BSA plus 10% normal goat serum (blocking solution) to reduce non-specific binding. The grids were then transferred to a 35 µl drop of the primary antibody (diluted 1:20 in the blocking solution). Sections were incubated with diluted primary antibodies for 2 h. Next, grids were blotted and rinsed four times on a drop of Tris buffer for 5 min. Then the sections were blotted and transferred to a drop of the blocking solution for 5 min. They were transferred to a 35 µl drop of a 20% solution (as recommended by the supplier) of goat anti-rabbit IgG gold conjugate (Electron Microscopy Sciences) and incubated for 50 min. The sizes of gold particles conjugated to secondary antibodies were 6 or 10 nm. Single labeling was done with 6 or 10 nm gold conjugated IgG. Sections were subsequently washed in Tris buffer with 2.5 M NaCl for 10 min, rinsed in distilled water, and dried. The final step involved incubating the sections on a drop of a 0.5 % formaldehyde for 10 min and rinsing with distilled water followed by complete drying.

Double-labeling was done following a protocol described previously by Bendayan [5.42]. Briefly, following the above steps for one side of the grid, the grid was turned over and the same procedure was followed except that the primary antibody and the size of the gold particle were changed. The sections were counter-stained with uranyl acetate and Sato lead before examination with a Philips 410 Electron Microscope. In addition to the cross-reactivity procedures, other controls consisted of omitting the primary antibody step and testing for non-specific binding of the gold particles to the tissue.

Pituitary Cell Counts. To assess relative cell frequencies, ten grids per animal were viewed using transmission electron microscopy. One section per grid was used to count cells and to assess binding of the gold particles. Only cells sectioned through a nucleus were counted.

Results

Fixation of equine pituitary tissues in 4% cacodylate-buffered formaldehyde with 0.1% glutaraldehyde followed by low temperature embedding in LR Gold preserved ultrastructural characteristics of the tissue and subcellular organelles within individual cells (Figure 5.1). Immunocytochemical staining with the pre-absorbed primary antibodies followed by secondary IgG-gold complexed antibodies resulted in specific staining which was associated with the secretory granules (Figures 5.2, 5.3). Thus, the fixation and embedding procedures were compatible with retention of antigenic sites associated with primary antibody binding to PRL and GH.

Specificity of the Antibodies. Gold-conjugated secondary antibodies did not bind to secretory vesicles when primary antibodies were omitted (Figure 5.4). Furthermore, gold-conjugated secondary antibody did not bind when the primary antibodies were pre-absorbed by the homologous pituitary hormone (Figures 5.5, 5.6). Immunocytochemical staining with anti-bGH and anti-pPRL antibodies was not abolished or visibly reduced when primary antibodies were pre-absorbed with heterologous antigens (Figures 5.2, 5.3 inset). Results from tests on absorbtion of anti-GH and anti-PRL antibodies with bLH, bTSH, oFSH, and hACTH are not shown; however, these protein hormones did not visually affect immunocytochemical staining for GH or PRL.

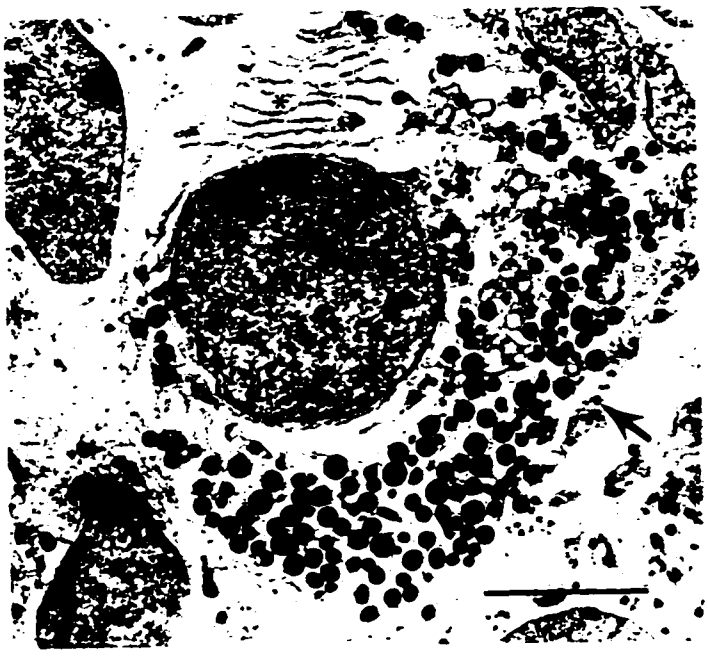

Figure 5.1. Pony pituitary tissue embedded in LRG showing a relatively well preserved somatotrope. Note that the small granules (arrow) in the adjacent cell are labeled for prolactin (6 nm immunogold; arrows). Rough endoplasmic reticulum (*), nucleus (N). Bar = 3 μm.

Figure 5.2. Characteristic ultrastructure and staining of Type I lactotropes in sections treated with anti-PRL antibody which were pre-absorbed with GH. Type I lactotrope cytoplasm stained with 10 nm gold particles. GH did not inhibit specific immunocytochemical staining of lactotropes. Note the variety in shapes of the lactotrope secretory granules, including a rod shaped one (arrow). Bar = .5 μm.

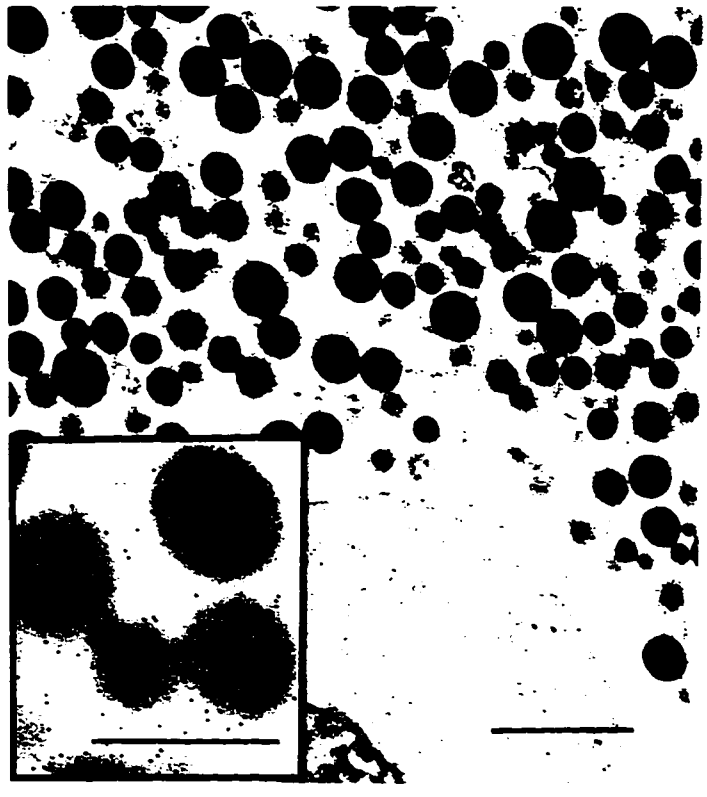

Figure 5.3. Typical morphology of somatotropes in sections treated with anti-GH antibody pre-absorbed with PRL. The somatotrope is labeled with 10 nm gold particles. Prolactin did not inhibit immunocytochemical staining of somatotropes. Note the uniformity in shapes of the secretory granules. Bar = 1 μm. Inset: An enlarged area of the cytoplasm. Bar = .5 μm.

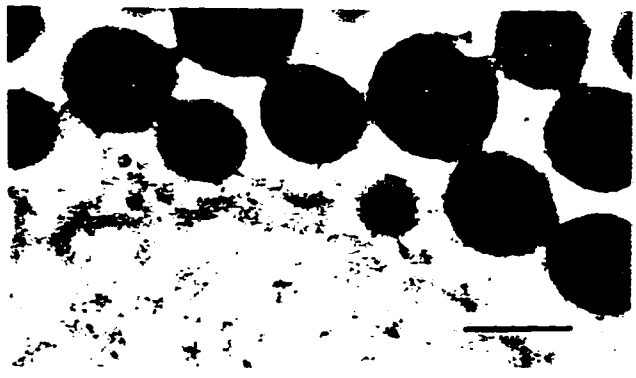

Figure 5.4. Somatotrope secretory granules in a pony pituitary. Only background binding of gold particles was observed following the omission of the primary antibody. Bar = .5 μm.

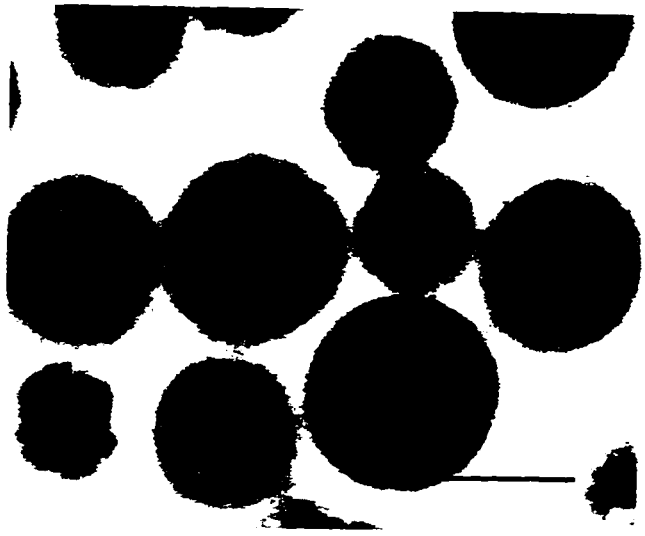

Figure 5.5. An enlarged area of cytoplasm in a somatotrope. The sections were treated with anti-GH antibody absorbed with GH. Only background binding of gold particles was found in these sections observed. Bar = .5 μm.

Figure 5.6. Type I lactotrope cytoplasm in sections which were treated with anti-PRL antibody pre-absorbed with PRL. Only background binding of gold particles was observed. Bar = 5 μm.

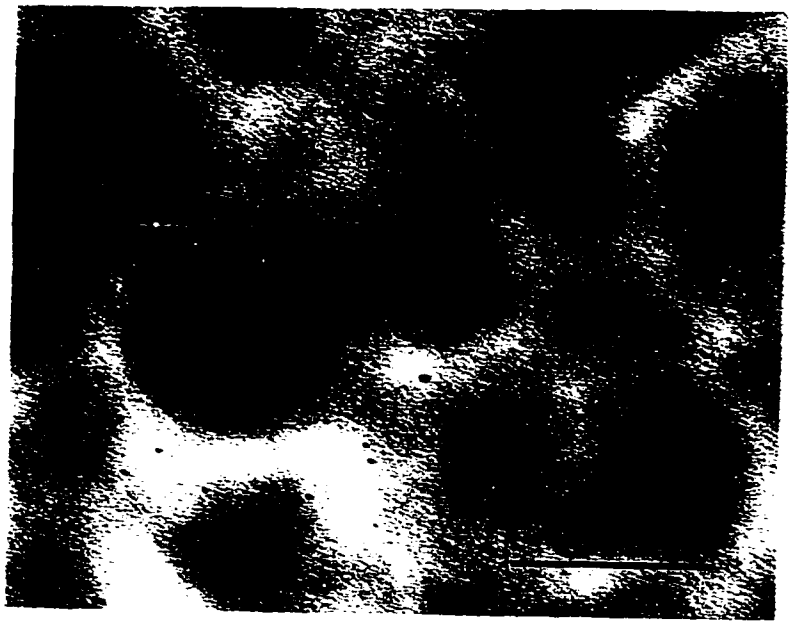

Figure 5.7. Ultrastructure of a mammosomatotrope in the pony pituitary: Double-labeling for PRL and GH in secretory granules. PRL = 6 nm, large arrow; GH= 10 nm, small arrow. Bar = .25 μm.

Therefore, pituitary cells stained with anti-GH antibody can be considered as GH containing-cells, cells stained with anti-PRL antibody as PRL containing-cell, and cells stained with both antibodies are considered to be mammosomatotropes (Figure 5.7).

Ultrastructural Morphology of the ir-PRL and ir-GH Cells. Pituitary cells labeled with anti-PRL antibodies were classified into two morphological subsets based on the size of secretory granules and cell size. Type I lactotropes commonly had eccentric nuclei, contained numerous large and dense polymorphic granules, and contained some rod shaped granules (Figures 5.2, 5.8). These cells had a large cytoplasmic volume relative to the size of the nucleus. Type I lactotropes were also readily recognizable at the ultrastructural level due to the characteristic morphology of the secretory granules. Type II lactotropes (Figures 5.9, 5.10) contained smaller granules which were located in the periphery of the cytoplasm. These cells have a small cytoplasmic to nuclear ratio and they were smaller in size than the Type I cells. Type I and Type II lactotropes did not stain for GH.

Growth hormone-immunoreactive cells possessed the largest secretory granules in the pituitary (Figures 5.11, 5.12). The granules were spherical in shape. Based on visual observation, somatotropes were medium-sized cells relative to other cells in the pituitary. The nuclei were generally eccentric and the cytoplasm was mostly filled with dense secretory granules (Figures 5.1, 5.3, 5.11, 5.13, 5.14). Epon-embedded sections revealed more detailed ultrastructure of the cells (5.8, 5.11, 5.13, 5.14). A few growth hormone secretory granules could be seen in the lumen of the Golgi apparatus (Figure 5.15).

Figure 5.8. Type I lactotrope in an Epon-embedded pony pituitary section. Note the polymorphism of the secretory granules (arrow). Nucleus (N). Bar = 2 μm.

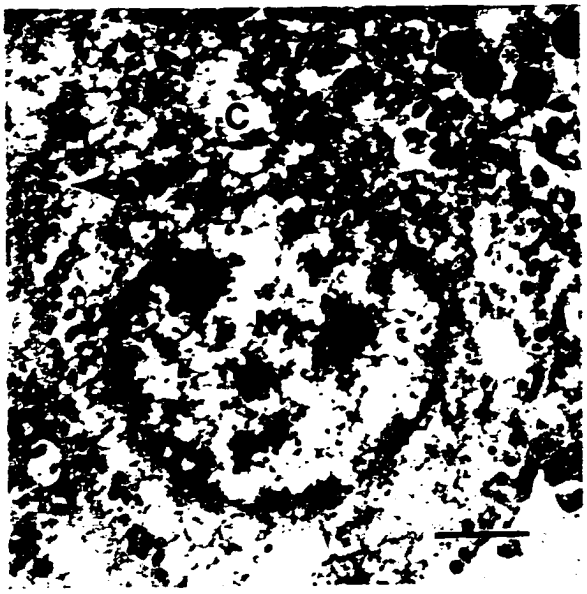

Figure 5.9. Type II lactotrope labeled with specific primary antibody and second antibody conjugated to 6 nm gold particles. Note the relatively small cytoplasmic (C) volume and small polymorphic granules in the periphery of the cell (arrows). Adjacent cell is a somatotrope (*). Nucleus = N. Bar = 1 μm.

Figure 5.10. Secretory granules in two Type II lactotropes adjacent to a somatotrope. Secretory granules of Type II lactotropes immunolabeled with 6 nm gold particles (arrows). Note the difference in the size of the secretory granules between the two cell types. Type II lactotropes stain exclusively for PRL. Somatotrope (S). Nucleus (N). Bar = 2 μm.

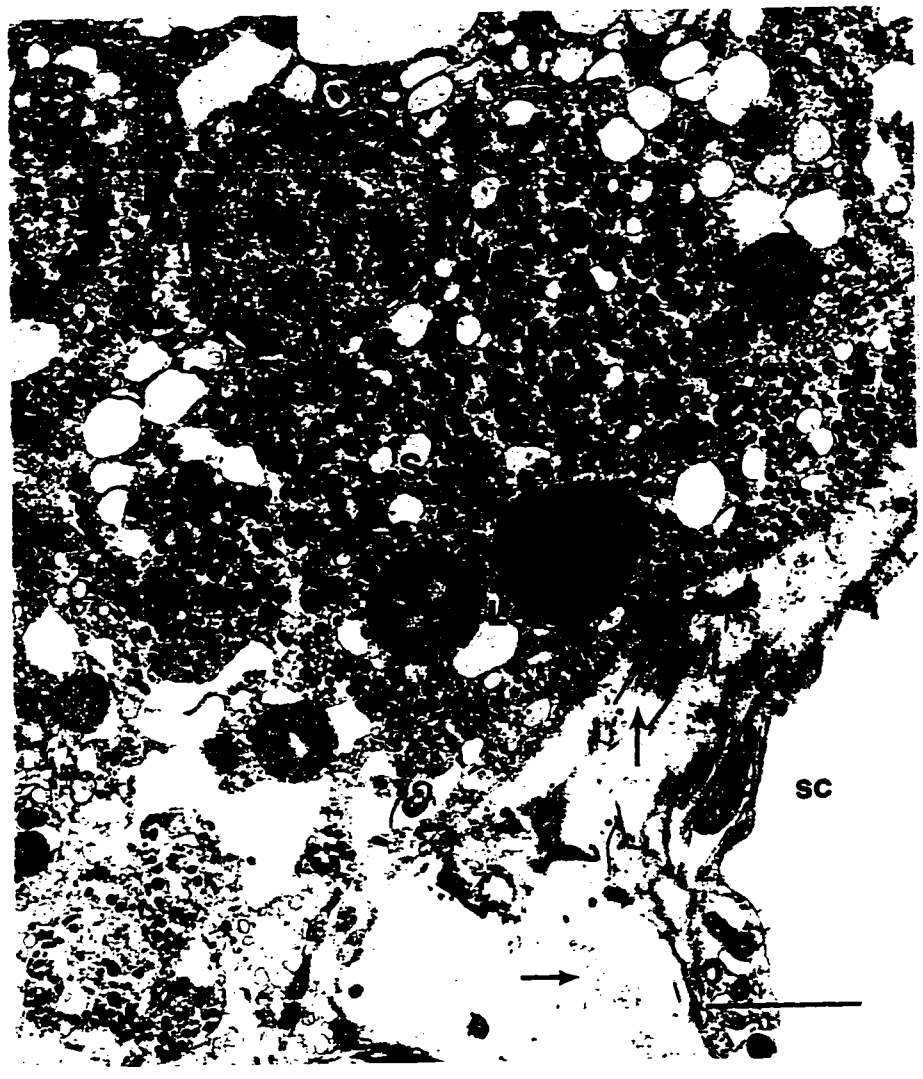

Figure 5.11. A somatotrope in an Epon-embedded pony pituitary section. The cytoplasm of another unknown cell type next to the somatotrope (S) contains numerous lysosomes (L). Nucleus (N), collagen fibers (arrows), and sinus cavity (sc). Bar = 2 μm.

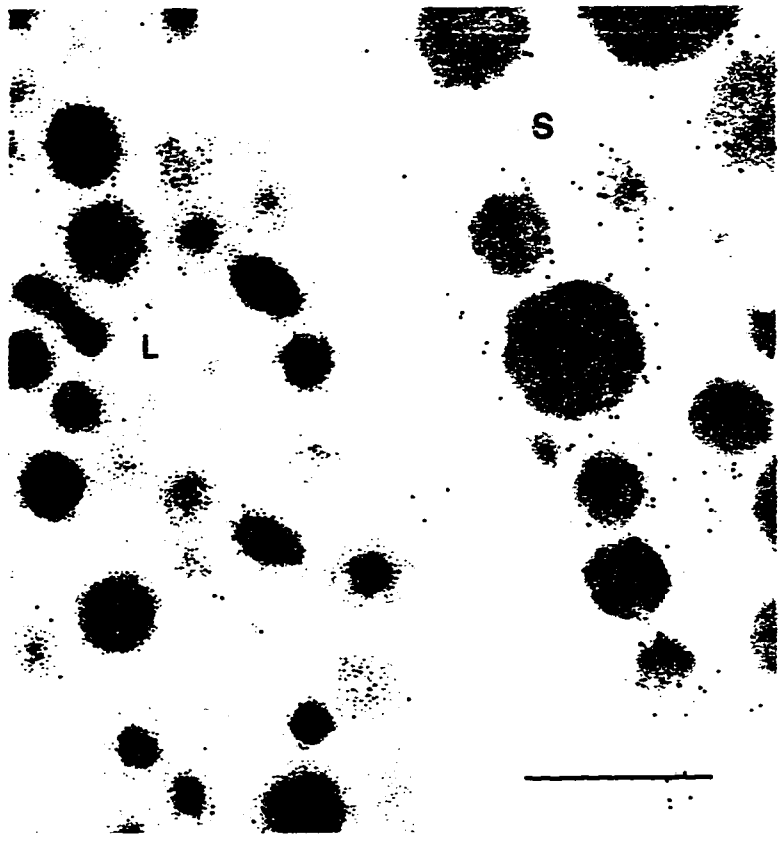

Figure 5.12. Double-labeled pony pituitary section showing a somatotrope next to a Type I lactotrope. The somatotrope (S) is labeled with 10 nm gold particles and the lactotrope (L) is stained with 6 nm gold particles. Bar = .5 μm.

Figure 5.13. Epon-embedded somatotrope in a pony pituitary section. Note the eccentric nucleus (N) and the homogeneous morphology of secretory granules. Bar = 3 μm.

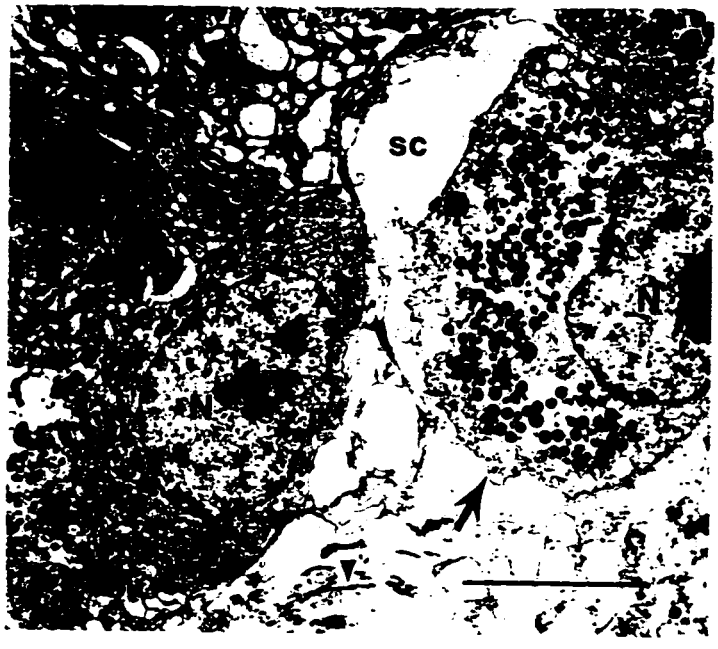

Figure 5.14. Epon-embedded pony pituitary tissue at low magnification: Somatotrope (arrow), sinus cavity (sc), collagen fibers (arrows head). A portion of a gonadotrope (*) is included in the field of this micrograph. Cell of unknown type (u). Nucleus (N). Bar = 5 μm.

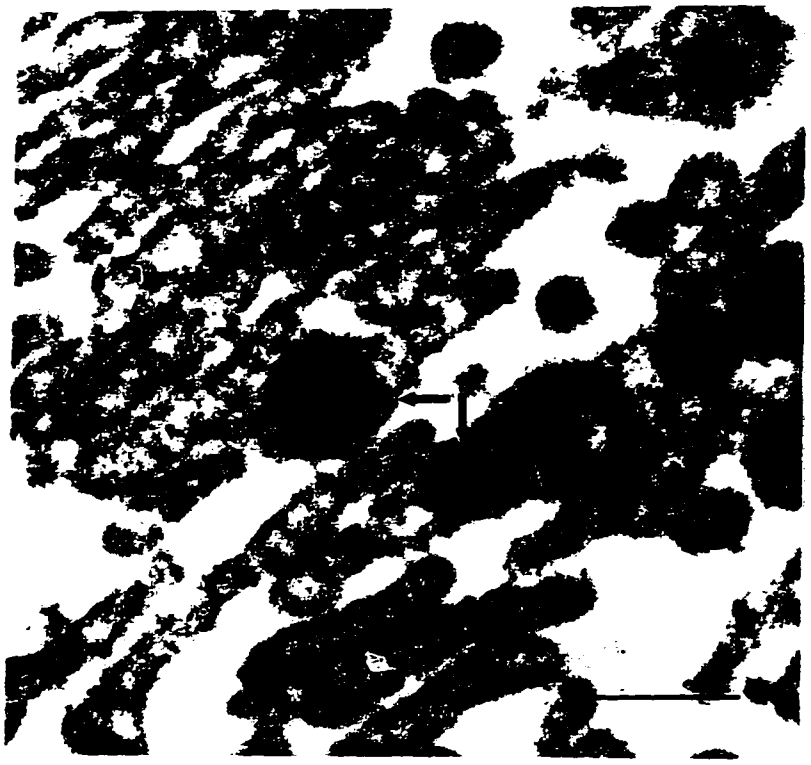

Figure 5.15. High magnification of secretory granules in the Golgi of a somatotrope (arrows). Bar = .5 μm.

Double-labeling revealed the presence of individual pituitary cells that stored both PRL and GH (Figure 5.7). These cells are generally known as mammosomatotropes. They are similar in size to somatotropes and Type I lactotropes. Granules in mammosomatotropes appeared similar to the large polymorphic granules in Type I lactotropes. Due to the similarities between mammosomatotropes and Type I lactotropes, it was not possible to differentiate these cells in Epon-embedded tissues. Double-labeling also revealed that lactotropes and somatotropes can be found next to each other (Figure 5.12). Different cellular junctions such as gap junctions and desmosomes were found between somatotropes and other cell types (Figures 5.16 A, B).

Cell Numbers and Reproductive Status of the Mares. Ovarian structures in the three animals included in this experiment and the plasma progesterone concentrations suggested that pony A was in the follicular phase, pony B was in the mid-to late luteal phase, and Pony C was in the early follicular phase (Table 5.1). The percentages of pituitary cells stained for only GH or PRL, and for both hormones are presented in Table 5.2. The numbers of cells stained for GH or PRL were 11 to 26% and 5 to 16% of total cells in the pituitary, respectively. Mammosomatotropes comprised about 7 to 17% of the total number of pituitary cells. Collectively, these three cell types, all together accounted for about 26 to 48% of the total number of pituitary cells counted in each animal.

Discussion

To our knowledge, this is the first description of the ultrastructural characteristics of ir-somatotropes and ir-lactotropes in the equine pituitary. These experiments

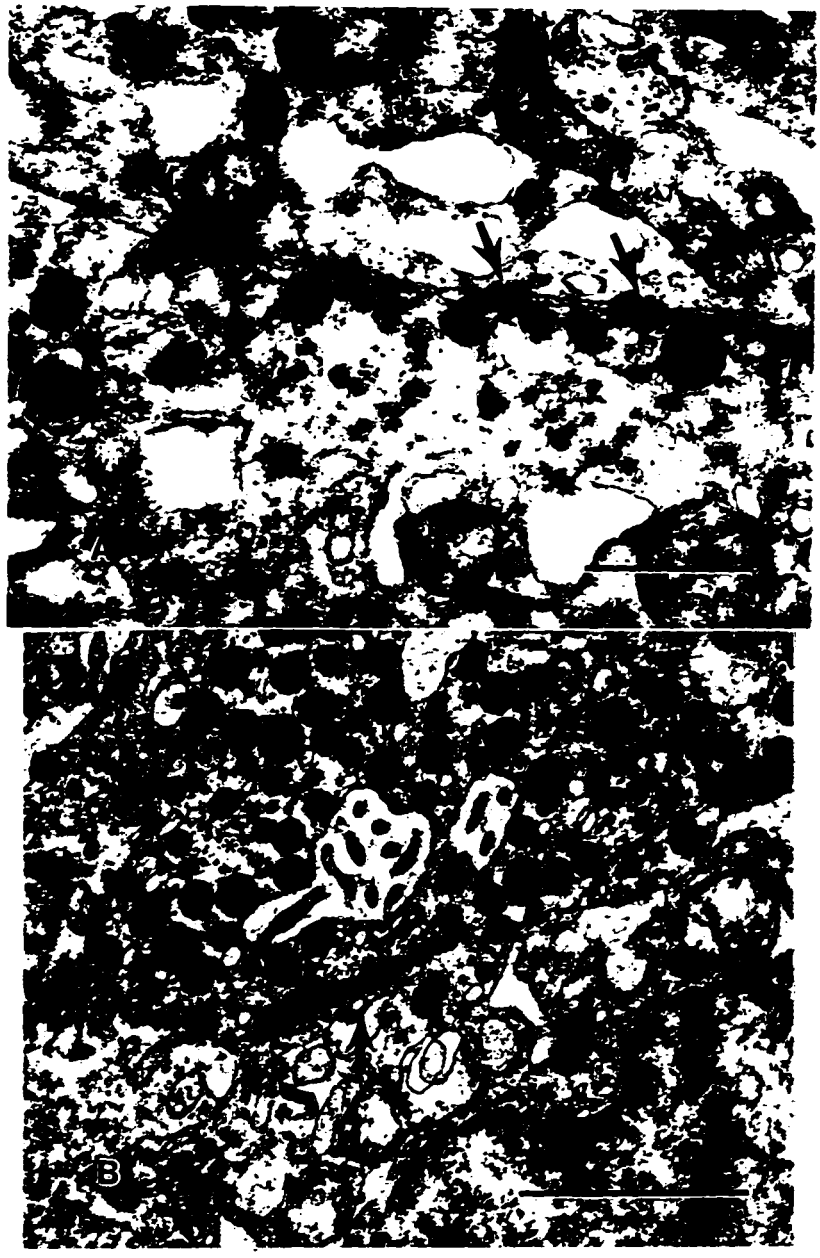

Figure 5.16. Presence of junctional complexes in a pony pituitary. A: Desmosomes (arrows) between a somatotrope and a mammosomatotrope or a Type I lactotrope. Bar = 5 μm. B: Gap junction (arrow) between two somatotropes. Bar = 1 μm.

Table 5.1. Reproductive status and the ovarian structures present at the time of slaughter.

Pony	Slaughter date	Right ovary	Left ovary	Plasma Progesterone (ng/ml)
A	8/3/95	20 mm follicle	40 mm follicle	1.5
B	5/4/94	45 mm follicle 28 x 25 mm CL	40 mm follicle	33.8
C	5/4/94	25 mm follicle	12 mm follicle	2.1

Table 5.2. Relative proportion of the GH and/or PRL containing cells in equine anterior pituitaries.

Pony	Total cells	% GH	% PRL	% Both	% Total
A	321	11.0	8.7	6.5	26.2
B	212	12.7	16.0	10.3	39.2
C	121	26.4	5.0	16.5	47.9
Average	218	16.7	9.9	11.1	37.8

Percentages are calculated by dividing the number of ir-cells by the total number of pituitary cells counted in a sample. Only cells with a nucleus were counted.

demonstrate that PRL and GH antigenicity and ultrastructure were preserved when low concentrations of buffered-glutaraldehyde and formaldehyde were used for tissue fixation and tissues are embedded at low temperature in a water miscible resin (LR Gold).

Several reports have classified lactotropes into two or three types based on morphology of the secretory granules and size of the cells [5.9, 5.15, 5.22]. In these ponies, cells which stained for PRL were distinguished into two different types based mainly on the size of the granules and the size of the cell itself. Type I lactotropes and their granules were larger than Type II cells and secretory granules in Type II cells. It remains to be determined whether both of these cell types actively secrete PRL or whether they respond differently to secretagogues or inhibitors.

An electron microscopic study done by Mikami [5.14] indicated that large lactotropes in the bovine pituitary gland were round or oval in shape; these cells contained large, dense granules, which were spherical or polymorphic. Ultrastructural studies by Heath [5.43] indicated that secretory granules of lactotropes in bovine pituitaries have the largest diameter when compared to granules in other pituitary cell types. Results from immunohistochemical studies of Dacheux and Dubois [5.15] with bovine pituitary also showed that PRL-secreting cells contained large secretory granules. However, these authors emphasized that the granules in lactotropes were smaller in diameter than the secretory granules in somatotropes. In goats [5.13], lactotropes contain the largest secretory granules of all pituitary cells . In equine pituitary glands, the secretory granules in lactotropes varied greatly in size. Nevertheless, the

granules in equine somatotropes were the largest in size and the most uniform in shape compared with secretory granules in other pituitary cell types.

Storage and secretion of GH and PRL are typically considered to be a function of two distinct cells in the pituitary gland. However, there are reports that another cell type contains and secretes both hormones. In fact, 26% of cells in the pituitary of lactating cows contain both ir-GH and PRL [5.16, 5.17]. Thorpe et al. [5.19] reported that only 2 out of 1800 cells counted contained both GH and PRL in sheep pituitaries. In a similar study, Thorpe and Wallis [5.20] cultured sheep pituitary cells and reported that 3% of the cells stained for both GH and PRL. However, by the end of the 96 hours of culture, <0.2% of the cells contained both PRL and GH. The decrease in the mammosomatotropic cell population was presumed to be due to cell culture or to the absence of hypothalamic factors necessary to maintain normal pituitary cell functions. Mammosomatotropes have also been found in mice [5.44], rats [5.33, 5.34, 5.45], musk shrews [5.45], and cows [5.33, 5.34].

There seems to be a distinct subcellular localization of different hormones in granules found in ir-mammosomatotropes. The study in musk shrews and rats showed that there are two types of co-localization patterns for GH and PRL. In one, GH and PRL were packaged separately. These were found in pregnant animals. In the second localization pattern, both hormones are packaged in the same secretory granule [5.45]. Fumagalli and Zanini [5.16] reported the presence of multinucleated mammosomatotropes in bovine pituitary glands. These authors also reported that GH and PRL are packaged in three different ways, with the hormones evenly mixed together

in the same granule, hormones found in different granules in the same cell, and with PRL and GH found within the same granules but segregated in different portions of the granule. In the present study, double-labeling demonstrated that both PRL and GH are evenly mixed together in one secretory granule. The mammosomatotropes comprised 6.5 to 16.5% of the cells counted in the three different gonadally intact mares. This percentage is in the range reported for the incidence of cells containing both GH and PRL in various domesticated species [5.16, 5.17, 5.19, 5.20].

The close association of lactotropes and somatotropes and their ability to interconvert apparently starts early in life. Hoeffler et al. [5.46] reported on ontogeny of GH and PRL cells in anterior pituitary cell cultures of neonatal male and female rats and found that GH secreting cells are firmly established by the time PRL secretors appear (between day 3 and 4 of postnatal life). By performing a reverse hemolytic plaque assay, these authors also found that the initial PRL secretors also secreted GH. This raised the possibility that PRL cells could arise from GH cells during development. Similar findings with regard to the ontogeny of these cells have been reported in other animals including humans [5.27], sheep [5.47, 5.48], pigs [5.49, 5.50], and mice [5.51]. Although, these cells might have a common stem cell, apparently they have evolved to respond differently to external and internal cues.

Thompson et al. [5.52, 5.53] studied the effects of several stimuli in horses and reported that GH secretion is stimulated by stimulation of the sympathetic nervous system, treatment of the animals with TRH did not elicit a GH response, and GHRH did not stimulate PRL release. These researchers did not find any correlation between PRL

and GH secretion in horses. Thus, the physiological significance of equine mammosomatotropes with granules containing both ir-GH and ir-PRL is not clear at the present time. It may be that ir-hormone detected immunocytochemically in these cells is not identical to radioimmunoassay hormone concentrations. However, results from hemolytic plaque assays of pituitary cells form other species suggests that simultaneous secretion of GH and PRL may occur in response to certain stimuli [5.36, 5.37]. Hemolytic plaque assays with equine pituitary cells and in situ labeling of mRNA for GH and PRL in equine pituitary cells would help to clarify whether ir-mammosomatotropes synthesize and/or secrete GH and PRL in a coordinated fashion.

In summary, the present study has confirmed that horses are similar to several other species in that cell populations that stain for GH and PRL can be divided into three types. One cell group stores both ir-hormones and the other two cell groups store a single ir-hormone. This basic, yet essential, information can be used to further investigate changes in the ratio of lactotropes and somatotropes that may occur during the anovulatory season. Immunocytochemistry, in situ hybridization, and hemolytic plaque assay can also be utilized to study the exact role of somatotropes in prepubertal and adult horses.

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CHAPTER VI

EFFECTS OF ESTRADIOL-17β AND DIHYDROTESTOSTERONE ON SOMATOTROPES AND LACTOTROPES IN EQUINE PITUITARIES: AN IMMUNOCYTOCHEMICAL STUDY

Introduction

Boockfor et al. [6.1] utilized a reverse hemolytic plaque assay with pituitary cell cultures from male rats in order to provide the first direct evidence that estradiol caused a marked increase in the prevalence of immunoreactive (ir)-mammosomatotropes and a concomitant decrease in the relative proportion of cells that released growth hormone (GH). These authors suggested that estradiol may convert cells that release only GH to those that release both GH and prolactin (PRL). Other studies have shown considerable fluctuation in number of cells which contain GH, PRL, or both hormones throughout the annual reproductive cycle of bats and musk shrews and during the transition from early pregnancy through the end of lactation in rats [6.2-6.4]. Other reports [6.5, 6.6] have further documented the effects of estradiol in modulating the proportion of somatotropes, lactotropes, and mammosomatotropes in culture or during different phases of the estrous cycle.

There are several reports concerning PRL secretion and storage in horses [6.7-6.14]. Johnson [6.7] found no change in PRL secretion throughout the estrous cycle of mares. Worthy et al. [6.13] reported brief increases in PRL secretion at the end of diestrous in three pony mares, yet these authors concluded that the exact pattern of plasma PRL concentrations during the estrous cycle was not clear. A study done by Thompson et al. [6.14] reported that treatment of ovariectomized (OVX) pony mares

with estradiol increased pituitary content of PRL, but this treatment did not increase serum PRL concentration. These authors also reported that treatment of OVX pony mares with dihydrotestosterone (DHT) or testosterone increased the PRL response to exogenous thyrotropin releasing hormone (TRH), even though neither androgen had any effect on daily secretion or storage of PRL [6.14].

Results with respect to the effects of estrogens and androgens on GH are controversial. Hassan et al. [6.15] reported that sex steroids did not affect basal release of GH, but androgens increased the responsiveness of somatotropes to growth hormone-releasing hormone (GHRH). Other reports have shown similar effects of androgens on basal and GHRH-induced GH release in rats [6.1, 6.16, 6.17]. However, administration of testosterone to juvenile male rhesus monkeys [6.18] resulted in an increase in plasma PRL, pituitary PRL content, and the number of ir-lactotropes. In vivo treatment of primates [6.19] and in vitro treatment of sheep pituitaries [6.20] with steroids had no effect on GH secretion. In order to gain a better understanding of steroid effects on equine pituitary cells, the present study used immunocytochemistry to differentiate and compare somatotropes, lactotropes, and mammosomatotropes in control and steroid-treated OVX pony mares. Steroids tested for an effect on these cell types included estradiol and DHT, a nonaromatizable androgen.

Materials and Methods

Animals and Treatments. Twelve adult (6 to 12 years of age) long term (>2 years) OVX pony mares weighing between 168 and 323 kg were kept on pasture and fed grass hay as needed to maintain good body condition. The mares were randomly allotted

to one of three treatment groups (n = 4/group) which included: 1) corn oil (control, 1 ml); estradiol (Sigma Chemical Co., St. Louis. MO), 35 µg/kg bodyweight; and DHT (Sigma Chemical Co.), 150 µg/kg bodyweight. All injections were given subcutaneously in the neck region. Treatment injections were repeated daily at 0800 for 21 days. Because of the time needed for processing tissues for electron microscopy and homogenization of the freshly collected tissue, one animal from each treatment group was started on treatment on one of four consecutive days and all animals were sacrificed on day 22. The experiment was performed between July 11 and August 4, 1994.

Daily blood samples were collected via jugular venipuncture immediately prior to the daily treatment or control injections. These samples were drawn into heparinized tubes; plasma was harvested by centrifugation and stored at -15°C for later analysis.

Pituitary Preparation for Hormone Analysis. Pituitaries were removed within 7 to12 min following euthanasia by electrocution. Tissues were rinsed with saline and cut in half, mid-sagittaly. One half was placed in ice-cold .01 M phosphate buffered saline (.15 M NaCl; PBS). This tissue was stripped of its surrounding capsule. The neural lobe was then separated from the anterior pituitary (AP) and the halved AP was weighed. The AP was homogenized in 100 ml of PBS in a Waring 7011 blender (Waring Prod. Div., Dynamics Corp. of America, New Hartford, CT) at maximum setting for 30 sec. Another 100 ml of PBS was used to rinse out the blender. The homogenates were mixed well and 10 ml aliquots were centrifuged at 1200 x g for 30 min at 4°C. The resulting supernatants were harvested and frozen. Pituitary homogenates were diluted 1:500 in PBS containing 0.1% gelatin. Concentration of PRL in plasma samples and GH

and PRL in pituitary homogenates were measured by RIA as described previously [6.21, 6.22].

Tissue Preparation for Electron Microscopy. Following a rinse with saline, the other half of AP was placed in freshly prepared 4% formaldehyde (Electron Microscopy Sciences, Fort Washington, PA) plus 0.1% glutaraldehyde (Polysciences, Warrington, PA) buffered with 0.1 M sodium cacodylate (pH 7.3; Electron Microscopy Sciences). While in the fixative, the pituitaries were freed of the surrounding capsule and the AP was removed from the neural lobe. The pituitary tissues were then cut into approximately 1 mm^3 pieces and fixation continued for 2 h. Following fixation, ten randomly selected pieces from each animal were removed and cut into smaller pieces; these were used for embedding. Tissues were washed several times in 0.1 M sodium cacodylate buffer to remove any residual aldehyde. Tissues were then dehydrated in 50% ethanol (EtOH) for 15 min, 70% EtOH for 20 min, and 90% EtOH for 30 min. Some of the tissue pieces were processed for ultrastructure study using routine Epon-Araldite embedding for transmission electron microscopy as previously described by Poolsawat [6.23]. Tissues used for immunocytochemistry were infiltrated and then embedded with 60% LR Gold (LRG; Electron Microscopy Sciences) monomer : 40% EtOH for 30 min; 70% LRG : 30% EtOH for 30 min; 100% LRG monomer for 2 h; 100% LRG monomer plus 0.1% benzil (initiator) for 2 h; and finally overnight in 100% LRG monomer plus initiator. All procedures were performed at room temperature. The tissues were then embedded in small gelatin or large BEEM® capsules (Electron Microscopy Sciences). The LRG was polymerized under UV light at -15°C for 24 to 36 h. Ultrathin, 80-90 nm

light gold-colored sections were cut on a Sorvall MT2-B ultramicrotome and sections were mounted on 400 mesh gold grids.

Immunogold Labeling. Sections were first incubated for 10 min on a drop of 0.05 M Tris (pH 7.3) containing 0.02 M glycine to quench any aldehyde left in the tissue. They were then incubated, for 30 min, on a drop of Tris buffer containing 1% BSA plus 10% normal goat serum (blocking solution) to reduce non-specific binding. The grids were transferred to a 35-µl drop of the primary antibody (diluted 1:20 in the blocking solution). Specificity of the primary antibodies has been characterized [6.24] and sections in the present study were incubated with hormone-specific primary antibody-fractions for 2 h. Next, grids were blotted and rinsed four times on a drop of Tris buffer for 5 min each. Then the sections were blotted and transferred to a drop of the blocking solution for 5 min. They were then transferred to a 35-µl drop of a 20% solution (as recommended by the supplier) of goat anti-rabbit IgG- gold conjugate (Electron Microscopy Sciences) and incubated for 50 min. The sizes of the gold particles used were 6 or 10 nm. Single labeling was done with either 6 or 10 nm gold conjugated IgG. The sections were washed in Tris buffer with 2.5 M NaCl for 10 min and rinsed in distilled water. The final step involved incubating the sections on a drop of 0.5 % formaldehyde for 10 min and rinsing with distilled water followed by complete drying.

Double-labeling was done following a protocol described previously by Bendayan [6.25]. Briefly, following the above steps for one side of the grid, the grid was turned over and the same procedure was followed except that the primary antibody and the size of the gold particle on the secondary antibody were changed. The sections were counter-

stained with uranyl acetate and Sato lead before examination with a Philips 410 Electron Microscope. Antibody specificities and cross-reactivity as well as immunocytochemistry controls have been reported elsewhere [6.24].

Statistical Analysis. Data consisting of repeated measurements taken over time were analyzed by ANOVA that accounted for the repetitive nature of blood sampling. Hormone concentrations in daily samples were calculated as a percentage of the pretreatment concentration and the least significant means (LSM) test was used to compare post-treatment means to 100%. Non-repetitive data were analyzed by one-way ANOVA; means for each treatment were compared to controls by the LSM test. All data were analyzed on PC-SAS [6.26].

Results

Plasma Hormone Concentrations. Pretreatment values (mean ± SE) for PRL were: Group 1/vehicle, 24.8 ± 20.7 ng/ml, Group 2/estradiol, 35.8 ± 34 ng/ml, and Group 3/DHT, 41.7 ± 22.6 ng/ml. Daily PRL secretion was very variable among the three treatment groups (Figure 6.1). There was no overall treatment affect ($P > .1$) on plasma PRL concentrations. Due to the episodic nature of GH secretion, one daily blood sample would not have adequately tested any increase or decrease in GH secretion; therefore, blood samples were not analyzed for GH concentrations.

Hormone Content in the Pituitary. Estradiol treatment caused an eight-fold increase ($P < .05$) in pituitary PRL content compared to pituitary PRL content in the control and DHT-treated groups (Figure 6.2). There was no significant ($P > .05$)

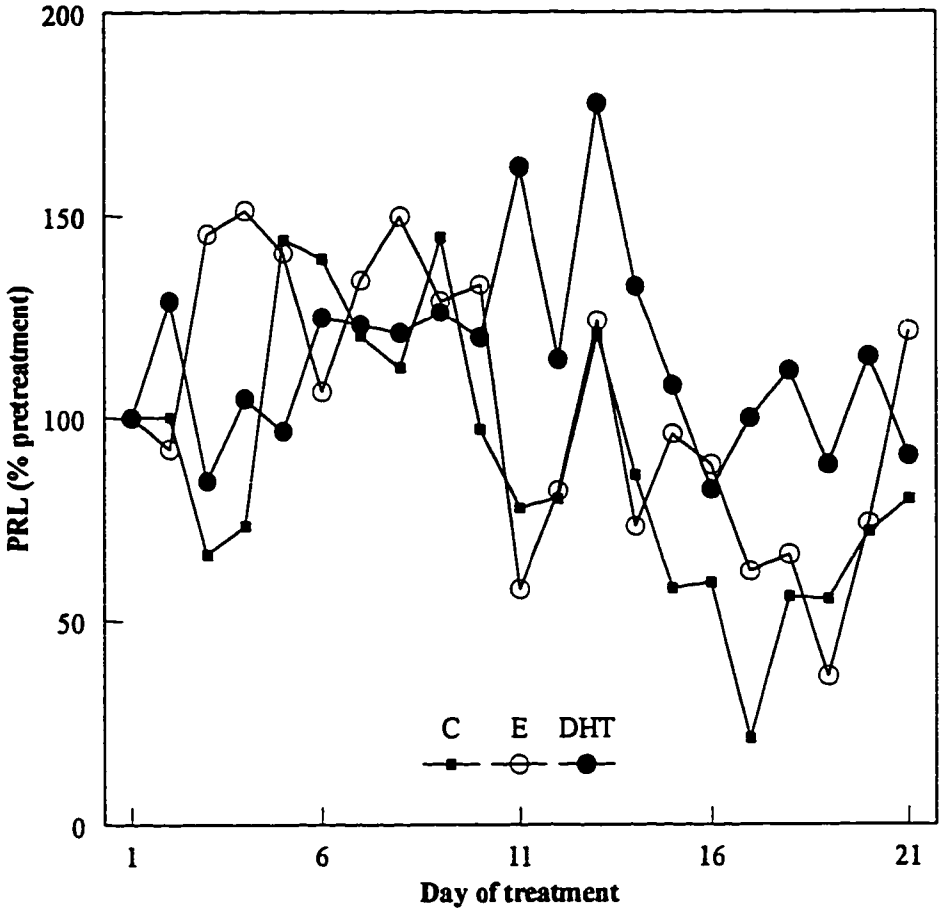

Figure 6.1. Plasma concentrations of PRL in control and steroid-treated OVX mares. Mean daily concentration of PRL as a percentage of pretreatment values. Mares were administered oil (C), estradiol (E), and DHT daily for 21 days. Data were analyzed by analysis of variance. There was no significant ($P > .1$) effect of treatment on plasma PRL.

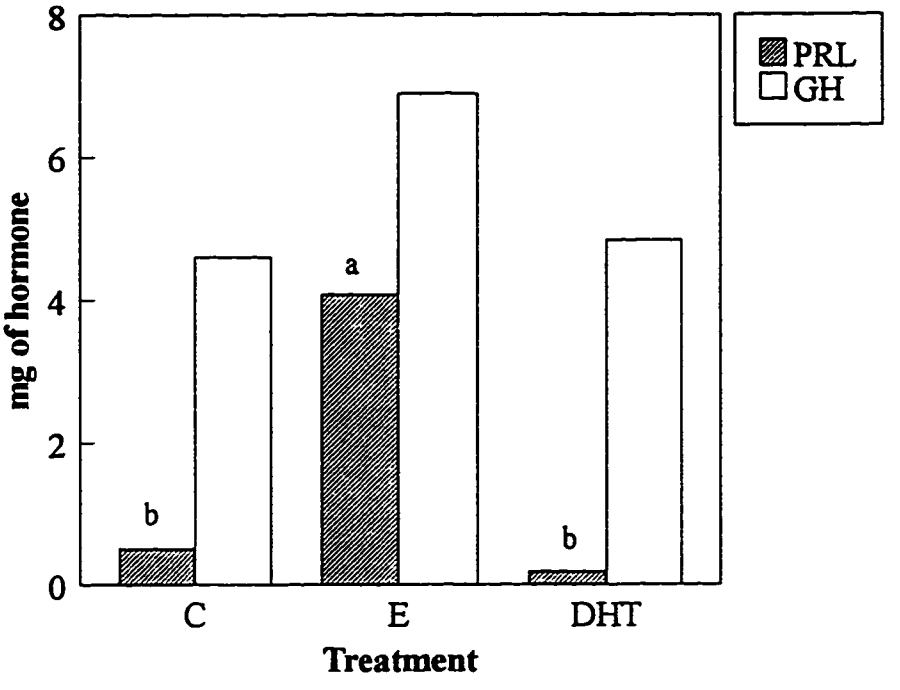

Figure 6.2. Pituitary content of GH and PRL in control and steroid-treated OVX mares. Mares were treated with oil (C), estradiol (E), or DHT daily for 21 days. For PRL, means with different letters were significantly ($P > .1$) different. There was no effect ($P > .1$) of treatment on pituitary GH content.

difference in pituitary PRL content for the control and DHT-treated groups. Growth hormone content of pituitary was not affected ($P > .1$) by any of the treatments.

Pituitary Cell Counts. Percentages of pituitary cells containing ir-GH, ir-PRL, and both ir-hormones are presented in Table 6.1. Total number of cells counted in each treatment group were: Oil, 466; estradiol, 628; and DHT, 599. The number of ir-PRL cells was significantly ($P < .05$) higher in the estradiol- treated group. Mammosomatotropes were also more prevalent ($P < .05$) in the estradiol-treated group. There was no effect ($P > .1$) of treatment on the percentages of ir-GH cells. In the control group, the percentages of ir-PRL and ir-GH cells were almost equal. These cell types collectively accounted for about 41% to 57% of the total number of pituitary cells counted. Mammosomatotropes included about 3 to 6% of the total number of pituitary cells in control and DHT-treated mares and 10% of the pituitary cells in estradiol-treated mares.

Ultrastructure of Somatotropes and Lactotropes. A detailed morphological characteristics of ir-somatotropes (Figure 6.3), ir-Type I lactotropes (Figure 6.4), and mammosomatotropes (Figure 6.5) has been reported [6.24]. Results from the present study indicated that the ultrastructure of various cell types in OVX pony mares is similar to that reported for gonadally intact mares. Based on visual observations there seemed to be a higher number of Type II lactotropes in estradiol-treated animals compared to the other two treatment groups (Figure 6.6). However, Type I and Type II lactotropes were not counted as distinct subsets in this experiment and additional data is needed to verify the differences in prevalence of these cell subtypes.

Table 6.1. Mean (M) and percentage (%) of cells stained for GH, PRL, or both hormones after treatment with vehicle, estradiol, or DHT.

	Control	Estradiol	DHT
Cell types	**M[a] (%)**	**M[a] (%)**	**M[a] (%)**
GH	25.8 (22.1)[1]	21.3 (13.5)[1]	36.8 (24.5)[1]
PRL	26.3 (22.5)[2]	53.8 (34.2)[3]	20.8 (13.8)[2]
Both	6.8 (5.8)[4]	15.5 (9.9)[5]	4.5 (3.0)[4]
Total	58.9 (50.4)	90.6 (57.6)	62.1 (41.3)

[a]Means represent average number of cells counted per grid (10 grids were counted for each horse).

The ± SE of the mean values for GH, PRL, and Both were 11.77, 7.67, and 2.04, respectively.

[1,2,3,4,5] Different superscripts in each row indicates a significant ($P < .05$) difference within that row.

Percentages are calculated by dividing the number of ir-cells by the total number of pituitary cells counted in a sample. Total number of cells counted in each treatment group were: Oil, 466; E_2, 628; and DHT, 599.

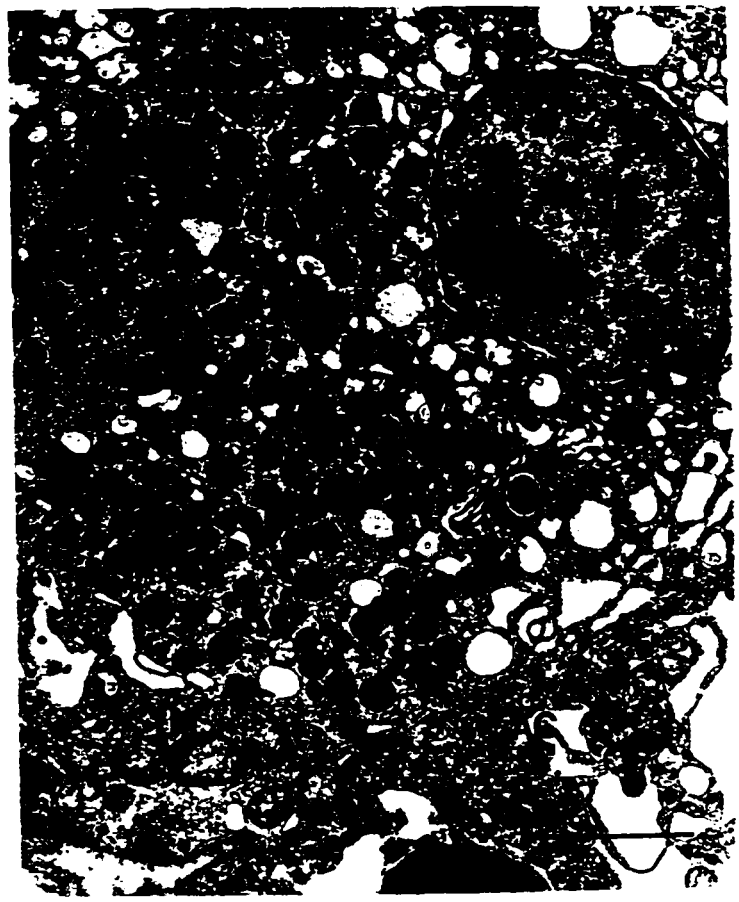

Figure 6.3. A somatotrope in an Epon-embedded pony pituitary section. Golgi body (arrow head), centriole (arrow), mitochondria (M), adjacent cell (AC), nucleus (N). Bar = 2 μm.

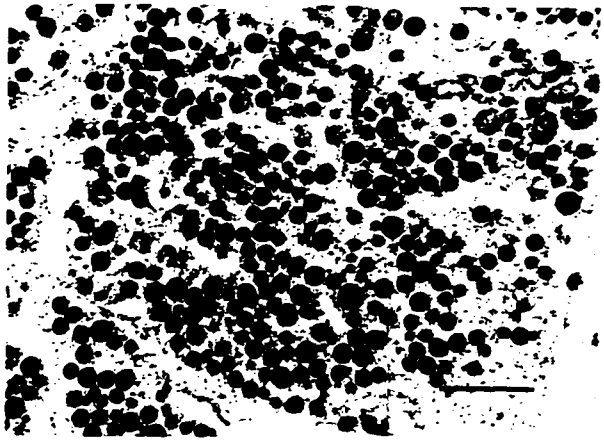

Figure 6.4. Equine pituitary lactotrope incubated with anti-PRL and anti-GH antibodies: Lactotropes only stained for PRL. PRL = 10 nm immunogold, GH = 6 nm. Bar = 1 μm.

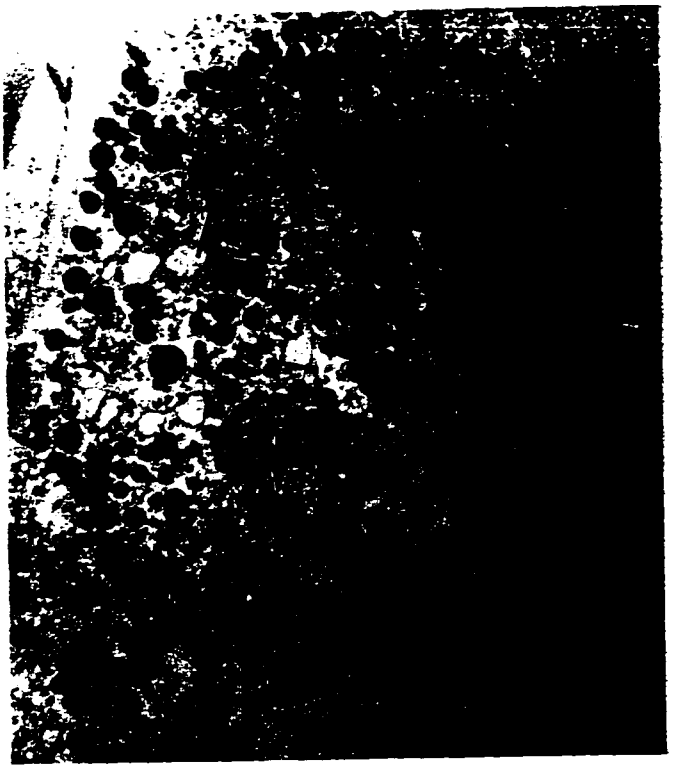

Figure 6.5. Epon-embedded mammosomatotrope in a pony pituitary section. Note the polymorphic granules (small arrows). Golgi body (arrow head), centriole (large arrow), nucleus (N). Bar = 1.5 μm.

Figure 6.6. A cluster of Type II lactotropes in the pituitary of an OVX pony treated with estradiol. Bar = 5 μm.

Discussion

Treatment of OVX ponies with estradiol caused a significant increase in cells stained for ir-PRL. Estradiol treatment also increased the total amount of PRL in the pituitary. At the same time, the proportion of cells that stained for both PRL and GH was increased by estradiol treatment. The increase in pituitary PRL and ir-cells which stained for PRL (lactotropes and mammosomatotropes) in estradiol-treated mares was not reflected in an increase in plasma PRL concentrations. Stratmann et al. [6.27] reported that following 21-day treatment of rats with estradiol there was a true transformation of somatotropes tc lactotropes. Later, Nogami [6.28] also showed that stimulation of newborn and adult rats with estradiol resulted in an increase in lactotrope populations. Finally, Frawley et al. [6.29] reported the presence of a distinct cell population in the pituitary that is capable of secreting both GH and PRL concomitantly, hence, these cells were referred to as mammosomatotropes. Later on, studies from the same laboratory showed that the fluctuation in number of lactotropes and somatotropes is influenced by gonadal steroids [6.5, 6.6] and that there is a bi-directional interconversion of these two cell types which depends upon hormonal cues [6.5]. Our study of pony pituitaries tends to support the conclusion that numbers of ir-lactotropes and somatotropes were affected by steroids and that there may be transdifferentiation [6.30, 6.31] of lactotropes and somatotropes. The augmentation in the total mammosomatotrope cell group in estradiol-treated mares could reflect cell division in the pituitary. This possibility was examined by Kineman et al. [6.31] where bovine pituitary cell cultures were subjected to several stimuli followed by reverse hemolytic plaque assay.

The results from that study indicated that acidophilic subpopulations of pituitary cells are capable of transdifferentiation and no significant cell division was involved. However, the authors reported that estradiol was incapable of increasing PRL in their culture system.

Thompson et al [6.14] reported a stimulatory effect of estradiol on storage of PRL. Yet, when challenged with TRH, there was no increase in PRL levels in the blood. In an experiment where the morphological characteristics of lactotropes and somatotropes were studied, we reported [6.25] that there are two distinct populations of lactotropes in the equine pituitary, Type I and Type II. Type I cells were characterized as being larger than Type II cells and they contained numerous large polymorphic secretory granules. Type II cells were smaller in size and had a large nucleus to cytoplasm ratio, and relatively small peripherally located secretory granules. In this study, based on visual observation, it appeared that there were more Type II lactotropes than Type I lactotropes in the estradiol-treated group whereas the proportion of these subsets of cells seemed to be equal in control OVX mares. Since a relative increase in plasma PRL concentration was not detected in this study it is a possible that these cells are not responsive to the secretagogues. This possibility is supported by experiments that did not detect a stimulatory affect of TRH on plasma PRL in OVX pony mares treated with estradiol for 21 days [6.14].

Estradiol effects on somatotropes are controversial and seem to be species-specific. In estradiol-treated rat pituitary cultures, Simard et al. [6.32] reported a 2- to 3-fold increase in GH release as well as the cellular GH content. Bethea and Freesh [6.33]

and Copeland et al. [6.19] reported that physiological doses of estradiol do not stimulate GH release in primates. However, Herbert [6.18] reported that administration of testosterone to juvenile male rhesus monkeys resulted in an increase in plasma PRL, pituitary PRL content, and the number of lactotropes. Our experiment with ponies did not detect an effect of estradiol on pituitary content of GH or the number of somatotropes. Kineman et al. [6.5] also reported that the relative proportion of somatotropes remained unchanged throughout the bovine estrous cycle.

Treatment of the OVX pony mares with DHT did not have any effect on plasma PRL concentrations. Treatment of OVX pony mares with androgens, and a subsequent challenge with TRH has been reported to increase plasma PRL concentrations [6.14]. Apparently androgens enhance the sensitivity of the PRL response to TRH. The lack of stimulatory effect of DHT on basal PRL secretion in this study was in agreement with the previous study [6.14]. Nogami [6.28] reported that testosterone administration to male rats decreased PRL secretion, whereas androgen treatment of juvenile monkeys caused an increase in pituitary PRL content and the number of PRL secreting cells [6.16]. Thus, androgen effects on basal plasma PRL concentrations and numbers of ir-lactotropes may be subject to species differences.

The exact role of GH and factors that might influence its secretion, storage, and release in the horse has not been thoroughly investigated. However, our laboratory has conducted a series of experiments to characterize the pattern of GH secretion and affects of various stimuli on GH secretion in horses [6.22, 6.34]. The findings from those experiments were that GH secretion is pulsatile in nature, the GH response to growth

hormone-releasing hormone (GHRH) was greater in stallions than in mares and geldings, acute physical exercise and exposure to an estrual mare increased plasma GH concentration, pharmacological inhibitors and/or stimuli for PRL secretion did not have any affect on GH, and GH levels were higher in males than in females. In this study, DHT treatment did not affect pituitary content of GH or the relative proportion of pituitary cells that stained only for GH. Testosterone has been shown to promote storage of GH in the pituitary of rats [6.35, 6.36] and increase the pituitary responsiveness to GHRH [6.17]. Our findings in the equine species are in agreement with other studies in that estradiol has a stimulatory effect on PRL and this is manifested at the pituitary level by increased pituitary content of PRL and increase numbers of pituitary cells which stain for PRL. Gonadal steroids do not seem to have any effect on GH content of the pituitary and the ratio of the three cell types described.

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CHAPTER VII

OVERALL SUMMARY AND CONCLUSIONS

The results from experiments included in this project demonstrate that fixation of equine pituitary tissues in cocadylate-buffered 4% formaldehyde with 0.1% glutaraldehyde followed by low temperature embedding in a water miscible resin resulted in ultrastructural preservation of the organelles and cell membranes. This approach also allowed for preservation of the antigens (hormones) in secretory granules, as was indicated by specific binding of the primary antibody preparations and gold-conjugated secondary antibodies. Our results agreed with the results of other researchers who have used a variety of species, in that the concept of exclusive "one-cell:one-hormone" producing cell types does not apply to certain subtypes of equine pituitary cells. Further, results from experiments described in Chapters III and V demonstrate that the ultrastructure and immunoreactivity of various subsets of equine pituitary cells are comparable to that described in most of the literature for other mammalian species. The findings form experiments described in Chapters IV and VI demonstrated the steroids may affect subsets of equine pituitary cells. These experiments also demonstrated that steroid treatment of OVX pony mares may affect the amount of ir-hormone in selected pituitary subset of cells, reciprocal transdifferentiation of the pituitary cells, and/or the total number of ir-cells as a percentage of the hormone-specific and chromophobic cells in the pituitary. These data collectively provide the basic information needed to conduct future studies on effects of exogenous treatments and physiological changes in specific subsets of equine pituitary cells.

VITA

Mohammad Saeed Rahmanian was born on October 14, 1960 to Esmael and Akram Rahmanian in Tehran, Iran. Saeed attended Hadaf High School until the 10th grade. In June 1977, he moved to the United States of America and graduated from Needham B. Broughton High School in Raleigh, North Carolina in May of 1978. After spending his freshman year at Campbell University, at Buies Creek, NC, he moved to the University of Tennessee, Knoxville, where he received his Bachelor of Science degree in Animal Science in May of 1982. In January of 1983, Saeed was accepted in the Department of Animal Science at the University of Wyoming to study ovarian physiology in sheep under the direction of Dr. William J. Murdoch. In the spring of 1986, Saeed received his Master of Science in Animal Science from University of Wyoming. In June of 1987, Saeed accepted an assistantship offer form Louisiana State University Dairy Science Department under Dr. J. D. Roussel. In the fall of 1989 he became a Research Associate at Louisiana State University working for Dr. D. L. Thompson, Jr. Saeed subsequently changed his department to Animal Science and continued his doctoral program in the area of reproductive physiology under Dr. Thompson. On August 27, 1990 Saeed was married to Melissa Lee Schakelford. Saeed completed his doctoral degree requirements in the Fall of 1996.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Mohammad Saeed Rahmanian

Major Field: Animal Science

Title of Dissertation: Immunocytochemical Localization of Gonadotropins, Growth Hormone, and Prolactin in Equine Pituitary

Approved:

Donald L Thompson Jr
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

J. Marcos Fernandez

Patricia A. Hansen

William Harvey

Date of Examination:

October 8, 1996